REMARKS

Claims 1-8, 12-17, and 21-39 are pending in the application. Claims 21-39 were withdrawn from consideration. Examination of the remaining claims, 1-8 and 12-17, is reported in the present Office Action. The examined claims were objected to, provisionally rejected under the judicially-created Doctrine of Obviousness-Type Double Patenting, and rejected under 35 U.S.C. § 112, first and second paragraphs, and 35 U.S.C. § 102(b) and (e). The objections and rejections are addressed below.

First, in response to the statement in the Office Action that the application is not in compliance with the sequence requirements of 37 C.F.R. § 1.821-1.825, applicants note that the Brief Description of the Drawings has been amended to include references to the sequence identification numbers that correspond to the sequences in Figures 1-3 and 7. This is consistent with M.P.E.P. § 2422.03, which states that "...when a sequence is presented in a drawing, regardless of the format or manner of presentation of that sequence in the drawing, the sequence must still be included in the Sequence Listing and the sequence identifier ("SEQ ID NO:X") must be used, either in the drawing or in the Brief Description of the Drawings" (emphasis added). Thus, based on this amendment, applicants respectfully submit that the application is in compliance with the sequence rules. In addition, applicants submit that this amendment addresses the objection to the specification based on the lack of sequence identification numbers in the Brief Description of the Drawings.

Applicants also note that the drawings were objected to because the legends (i.e., "Figure X") are illegible. In response, applicants enclose herewith formal drawings for this case.

The Office Action also notes that the priority claim must be added to the specification.

As is noted above, the specification has been amended in this manner.

The Examiner has maintained the previously made Restriction Requirement. Applicants respectfully disagree with the maintenance of this Requirement. First, applicants note that the Office Action states that the Examiner acknowledges "applicant's election with traverse of Group I, claims 1-8 and 12-17 to the extent of methods of treating or preventing by administration with a peptide of SEQ ID NO:15..." Applicants respectfully request clarification of this statement. In particular, it appears from this statement that the sequence of SEQ ID NO:15 may be being read by the Examiner as a limitation of all of the examined claims, not just those that include sequence identification numbers. Applicants respectfully submit that claims which do not include sequence identifiers should not be so limited, as that is not the way the claims were drafted. If applicants have not misunderstood the Examiner's statement concerning the applicability of SEQ ID NO:15 to all of the claims, they respectfully request that the Examiner provide them with a specific reference to an authority which states that it is acceptable for the Office to add limitations to claims in this manner.

Further regarding the Restriction Requirement, applicants note that the criteria for restriction in Markush claims (e.g., claim 8) are discussed in M.P.E.P. § 803.02. The requirements set forth in this section for maintaining the members of a Markush group in a single application are that the members must "(1) share a common utility, and (2) share a substantial structural feature disclosed as being essential to that utility." The members of the Markush groups of the present claims clearly meet these criteria. For example, in the case of the claims including multiple sequences, the peptide fragments having these sequences are all used as vaccine antigens to induce an immune response to β-amyloid, and thus share a common utility.

In all being immunogenic fragments of amyloid-β peptides, they all share a substantial structural feature as well. The possibility that they may have some separate, independent uses is not relevant, because the criteria cited above provide that they must share a common utility and a substantial structural feature, not all common utilities and all substantial structural features.

M.P.E.P. § 803.02 also states that "if the members of the Markush group are sufficiently few in number or so closely related that a search and examination of the entire claim can be made without serious burden, the examiner must examine all the members of the Markush group in the claim on the merits, even though they are directed to independent and distinct inventions" (emphasis added; also see M.P.E.P. § 803.01). As is discussed above, the members of the Markush groups of the present claims including multiple sequences are closely related, in being overlapping fragments of a single, short polypeptide. Moreover, the numbers of the members of the Markush groups are few. Thus, even if the members of the Markush groups were independent and distinct inventions, it would be incumbent upon the Examiner to search the full scope of the claims. Further, applicants submit that it is standard in the art to search a relatively longer sequence (e.g., a β-amyloid peptide sequence) and to obtain from the search hits that include shorter sequences (e.g., the sequences specified in claim 8) that have stretches of sequences that are identical to sequences within the larger sequence. Such a search could be done in the present case, to search the full scope of, e.g., claim 8, without undue burden. Applicants thus respectfully request that the Examiner reconsider the Restriction Requirement.

The objections and rejections are now addressed.

Double Patenting

Claims 1-8 and 12-17 were provisionally rejected under the judicially-created Doctrine of Obviousness-Type Double Patenting over claims 46-56, 58-64, and 66-109 of U.S. Serial No. 09/724,842. When the only rejection remaining in a case is an obviousness-type double patenting rejection, an application should be allowed to issue. M.P.E.P. § 822.01. In view of the amendments and remarks made herein, applicants submit that all of the grounds of rejection in this case, other than the obviousness-type double patenting rejection, have been met. Accordingly, the obviousness-type double patenting rejection should be withdrawn and the case allowed to issue.

Claim Objection

Claims 1-8 and 12-17 were objected to as reciting improper Markush groups. The Examiner supports this rejection by citing a passage from the M.P.E.P., § 803.02, which states "it is improper for the Office to refuse to examine that which applicants regard as their invention, unless the subject matter in a claim lacks unity of invention," (citations omitted) and that "...unity of invention exists where compounds included within a Markush group (1) share a common utility and (2) share a substantial structural feature disclosed as being essential to that utility." Applicants respectfully request that this objection be withdrawn.

First, applicants note that the only claims under examination that include Markush groups are claims 3-5, 8, 12, 14, and 17, and thus that this objection should not have even been considered with respect to the other examined claims (1, 2, 6, 7, 9-11, 13, 15, and 16).

Second, the elements in the claims that include Markush groups satisfy the criteria noted

by the Examiner in this rejection. In particular, claims 3, 8, and 17 each include within their Markush groups particular peptides of amyloid fibrils or proteins. As is discussed above in reference to the Restriction Requirement, these peptides are all used to induce an immune response to prevent or treat an amyloid-related disease, and thus share a common utility, and are all peptides of amyloid fibrils or proteins, and thus share a substantial structural feature that is essential to that utility. Thus, because the Markush groups of these claims meet the criteria for unity of invention in such a claim, this objection should be withdrawn.

Regarding claims 4, 5, 12, and 14, applicants note that these claims include Markush groups that specify variable N-terminal and C-terminal substituents for amyloid peptides. These substituents are chemical groups, e.g., alkyl groups, heterocyclic groups, acyl groups, alkoxy groups, and amino groups. Applicants note that it is standard for chemical patents to include claims that include Markush groups of chemical substituents that are far larger than these. It thus appears to be acceptable Patent Office practice to maintain such groups in chemical applications, and applicants respectfully submit that this standard should be applied in the present application as well. The elements of these Markush groups provide chemical modifications of the ends of the peptides, and the peptides themselves could be searched without these modifications (see above) with ease. Any hits could then be analyzed for the presence of the short lists of substitutions in these Markush groups.

Rejection under 35 U.S.C. § 112, first paragraph

Claims 1-8 and 12-17 were rejected under § 112, first paragraph as being overly broad. In particular, the Examiner states that the specification enables certain animal-based methods,

based on the teachings of Schenk et al., WO 99/27944, but does not enable the prevention or treatment of amyloid related disease, particularly in a human or with the breadth of the peptides claimed. This rejection is respectfully traversed.

The Examiner first refers to the exemplifications of Schenk et al., Nature 400:173-177, 1999, and states that the mouse model used by Schenk is not recognized as providing teachings that would be predictive of results that are consistent with the full scope of the claims, which include the treatment of humans. Applicants respectfully disagree.

Mouse models, such as that of Schenk, were widely used in the art at the time of the invention in studies carried out to characterize and test candidate treatments for amyloid-related diseases such as Alzheimer's disease. As another example of such a model, applicants submit herewith a paper by Chishti et al., J. Biol. Chem. 276(24):21562-21570, 2001 (a copy is enclosed), which describes a transgenic mouse model characterized by the deposition of β-amyloid and cognitive defects by the age of 3 months. The authors found that learning impairment in these mice was offset by immunization against Aβ42. Further, the authors refer to an earlier paper (Janus et al., Nature 408:979-982, 2000; a copy of the abstract is enclosed), which shows that such vaccination is also effective at reducing cognitive dysfunction and the deposition of cerebral fibrillar Aβ in the TgCRND8 murine model of Alzheimer's disease. In addition, the authors make note of several additional animal models of this disease (page 21563, left column). Further, another earlier paper (Morgan et al., Nature 408:982-985, 2000; a copy is enclosed) describes yet another transgenic mouse model of Alzheimer's disease, in which the authors found that vaccination with Aβ protects mice from learning and age-related memory deficits that normally occur in this model. Thus, it is clear that animal models of amyloid-related

disease were in use at the time of the present invention, and that those of skill in the art used these models in studies aimed at identifying and characterizing potential treatments of human disease. The fact that perhaps not each and every feature of such diseases may be mimicked in a particular model does not mean that the model does not have predictive value. Indeed, animal models such as those described in the cited papers have been widely used to test treatments for many diseases, just prior to testing in humans.

In further support of the position that the art teaches a lack of correlation of benefits shown in the mouse model to humans, the Examiner refers to Münch et al., J. Neural Transmission 109:1081-1087, 2002, stating that this paper shows that treatments effective in mice evoked neurotoxicity when practiced in humans. The teachings of the Münch paper do not show that the results obtained in the mouse model cannot be correlated with results expected in humans. Rather, it shows that in a small percentage (<5%) of patients vaccinated with an amyloid-β peptide in a clinical trial, central nervous system inflammation occurred, resulting in the halting of the trial. The study was not halted because it showed that the treatment did not work, and the paper provides no basis for concluding that the patients did not benefit from the treatment. In fact, the enclosed paper by Hock et al. (Neuron 38:547-554, 2003) shows that the patients did indeed benefit from the treatment. In particular, the Hock paper notes that after the suspension of the trials described in the Münch paper, a cohort of 30 patients with Alzheimer' disease who participated in the trials were followed. Hock found that patients who generated antibodies against β-amyloid showed significantly slower rates of decline in cognitive functions and activities of daily living, as compared to patients without such antibodies. This observation led Hock to conclude that "antibodies against β-amyloid plaques can slow cognitive decline in

patients with Alzheimer's disease," clearly showing that the vaccine approach to treating this disease is effective.

Further, Münch states on page 1082 that the companies carrying out the trial, Elan Corporation and Wyeth-Ayerst Laboratories, intended to proceed with a vaccine-based approach to therapy, providing validation for this approach. In fact, a recent press release (a copy is enclosed) from Elan states that the company plans to reinitiate human trials with a reformulated version of their vaccine. And it is not only these companies that are interested in continuing with such an approach, in spite of the initial clinical trial results. For example, referring to these studies, Cirrito et al., J. Clin. Invest. 112(3):321-323, 2003 (a copy is enclosed), state "[t]hough the first clinical trials for Aβ vaccination were halted due to CNS inflammation in a small subset of subjects, active and passive immunization strategies remain a viable potential therapy worth continued exploration." The Münch paper thus should not be interpreted as showing that vaccine approaches to treating amyloid-related diseases cannot be effective.

The Examiner goes on to state that the animal model system does not show that the treatment is effective to prevent the onset of disease. In response, applicants point to the experimental results described in Example II of the present application (pages 47 and 48). These experiments show that antibodies raised against Aβ peptides can block fibrillogenesis in the Thioflavin T assay and by electron microscopy. These results, showing that the process involved in the formation of amyloid deposits can be blocked by antibodies, certainly support applicants' position that the present methods are effective in preventing disease onset, in addition to treating ongoing disease.

The Examiner then goes on to reference papers showing the efficacy of certain peptides in

removing amyloid plaque burden (Lemere et al., Society for Neuroscience, Abstracts 25:519.6, 29th annual meeting, 1999; Schenk et al., Nature 400:173:177, 1999; Nordstedt et al., WO 97/21728 and U.S. Patent No. 6,331,440, and Kiessling et al., U.S. Patent No. 6,022,859), but states that these references do not show the relative ability of alternative peptides to achieve this effect.

In response, applicants refer to the accompanying Declaration of Francine Gervais, which was originally submitted in the parent case, U.S. Serial No. 09/724,842. The Declaration shows that peptides including D amino acids are effective in inducing antibodies against amyloid-β peptides, as well as in decreasing amyloid-β levels in the brain. Further, the Declaration shows that D peptides are unexpectedly better than corresponding L peptides. More specifically, the experiments described in the Declaration show that a D peptide including amino acids 10-22 of Aβ (BSA-(C)D10-22) induced high levels of antibodies in rabbits, and that these levels were substantially higher than those induced by a corresponding L peptide (BSA-(C)L10-22)(nearly 25,000 vs. undetectable). Similar results were obtained for another set of constructs including amino acids 10-22 of Aβ (KLH-(C)C10-22 vs. KLH-(C)L10-22). The experiments also show that a D peptide including amino acids 13-22 of Aβ (BSA-(C)D13-22) induced high levels of antibodies in rabbits, and that these levels were substantially higher than those obtained with a corresponding L peptide (BSA-(C)L13-22). The experiments in the Declaration further show that vaccination with a D peptide (D13-21-KLH) is effective in decreasing the levels of Aβ40 and Aβ42 in the brains of a transgenic mouse model of Alzheimer's disease, and that use of a D peptide is more effective than a corresponding L peptide (L13-21-KLH). Further, the results show that the D peptide is effective at increasing plasma levels of Aβ in immunized mice, to a

level that is greater than that obtained using a corresponding L peptide. These results show that peptides such as those of the present claims are effective.

The Office Action further states that protein chemistry is an unpredictable area of biotechnology, and refers to two papers to support the point that proteins with deletions, insertions, or substitutions may have structural and functional changes in biological activity and immunological recognition. The first reference, Skolnick et al., Trends in Biotech. 18(1):34-39, 2000, focuses on how genome sequencing projects have provided information on protein sequences, and states that such information is not sufficient to teach protein function, due to the complex folding, etc., required to achieve function. The reference does not mention the effects of structure on immunological recognition, and thus does not appear to be relevant to the present rejection.

The second reference, Jobling et al., Mol. Microbiol. 5(7):1765-1767, 1991, is cited for teaching that single amino acid substitutions in a protein (the B subunit of cholera toxin) produce proteins with differing conformations, immunological recognition, binding, and toxicity. With respect to immunological recognition, the paper makes note of substitutions at amino acid position 88 as resulting in decreased levels of immunoreactive protein. In explaining why substitutions at this position may have led to such a decrease, the paper states that it could be due to a defect at any step in the pathway from gene to protein (page 1763). For example, the paper states that the decreased levels could be due to degradation prior to or during secretion, folding into a conformation that is incompatible with secretion, proteolysis in the periplasm, or inability to fold into wild type conformation. Thus, the paper does not provide support for the position that mutations necessarily decrease immunological recognition, but rather points more to

mutations as affecting the endogenous protein from being produced and secreted properly. Further, tests are provided in the present application for determining whether a candidate peptide has the desired effects. In particular, Examples I and II describe *in vitro* assays for determining whether a peptide has anti-fibrillogenic activity. Thus, candidate peptides including mutations could easily be tested in such assays.

The Examiner goes on further in this rejection to state that Schenk et al., WO 99/27944, teaches the use of several peptides (Aβ 1-5, 1-12, 13-28, and 33-42), but only shows that one of them (Aβ 1-5) was effective at reducing plaque burden in PDAPP mice, and only in the cortex of the mice. Based on this, the Examiner concludes that even minor changes can affect the activity of peptides, and notes that the present specification does not teach which residues can be modified to retain activity. In reply, applicants repeat that Examples I and II of the present application provide details as to how to determine whether a particular peptide has antifibrillogenic activity. Thus, if one skilled in this art wanted to alter the sequence of a peptide and determine whether it would be effective in the methods of the invention, such assays could be carried out with the altered peptides. It is well accepted in the art that *in vitro* assessment of the anti-fibrillogenic activity of antibodies generated against a candidate peptide is a good predictor of its *in vivo* anti-amyloid activity using an animal model. Carrying out such *in vitro* assays certainly does not require undue experimentation.

Finally, the Office Action states that the claims recite the use of antigenic all-D peptides by their functional characteristics, and not by particular amino acid structure, and that the specification does not teach which peptides have the desired effects. The Office Action further states that the specification fails to provide suitable methodology for determining whether a

peptide may have such effects. Applicants respectfully disagree. As is discussed above, assays for testing the peptides are described in the Examples, on pages 47-48 of the specification. In particular, the Examples describe the induction of antibodies against candidate peptides and the testing of the antibodies in antifibrillogenic assays. Further, animal model systems were known in the art for testing such peptides. Thus, applicants have provided sufficient information for those of skill in this art to identify and characterize candidate peptides.

In view of the above, applicants respectfully request that the rejection under § 112, first paragraph be withdrawn.

Rejection under 35 U.S.C. § 112, second paragraph

Claims 1-8 and 12-17 were rejected under § 112, second paragraph as being indefinite in reciting the term "all D," which the Examiner states would be accepted in the art as meaning that every amino acid in the peptides are D amino acids. The Examiner thus concludes that the metes and bounds of the amino acids that are required to be D amino acids are indefinite. In the interest of expediting prosecution, applicants have amended the claims to specify that the peptides used in the claimed methods comprise at least 50% D amino acids. Support for this amendment can be found in applicants' definition of "all D" peptides, which is on page 26, lines 22-25 of the specification.

Rejections under 35 U.S.C. § 102

Claims 1-8 and 12-17 were rejected under § 102(b) as being anticipated by Nordstedt et al., WO 97/21728, and under § 102(e) over Nordstedt et al., U.S. Patent No. 6,331,440. These

rejections are respectfully traversed.

The rejection is based on the Examiner's position that the limitations of the present claims are taught in the Nordstedt references, which teach administration of L or D peptide binding sequences of β-amyloid to prevent or treat amyloid fibril formation, in light of the teachings of Schenk (supra) that such peptides are recognized as being capable of eliciting an immune response. Applicants respectfully disagree with this rejection.

The Nordstedt references provide no teaching of the use of amyloid peptides to induce an immune response, as is required by the present claims. Rather, these documents teach the use of such peptides to inhibit amyloid fiber formation by competitive inhibition. Schenk does not show that the peptides of the present claims are capable of eliciting an immune response, because the peptides of Schenk are L peptides, not the D peptides required by the present claims. Thus, because the cited references do not describe the present invention, this rejection should be withdrawn.

Claims 1-8 and 12-17 were also rejected under § 102(e) as being anticipated by Kiessling et al., U.S. Patent No. 6,022,859. This rejection is respectfully traversed.

Similar to the Nordstedt references, the Kiessling patent teaches peptides that disrupt β-amyloid aggregation. Kiessling does not describe the use of such peptides in immunization methods, and the fact that the Schenk reference teaches that L peptides are immunogenic is not relevant, as Schenk does not describe D peptides, as is required by the present claims. This rejection should therefore be withdrawn.

CONCLUSION

Applicants submit that the claims are in condition for allowance, and such action is respectfully requested. If there are any charges not covered or any credits, please apply them to Deposit Account No. 03-2095.

Respectfully submitted,

Date: October 23, 2007

Susan M. Michaud

Susan M. Michaud, Ph.D.
Reg. No. 42,885

Clark & Elbing LLP
101 Federal Street
Boston, MA 02110
Telephone: 617-428-0200
Facsimile: 617-428-7045

Neuron, Vol. 38, 547–554, May 22, 2003, Copyright ©2003 by Cell Press

Antibodies against β-Amyloid Slow Cognitive Decline in Alzheimer's Disease

Report

Christoph Hock,* Uwe Konietzko,
Johannes R. Streffer, Jay Tracy, Andri Signorell,
Britta Müller-Tillmanns, Ulrike Lemke,
Katharina Henke, Eva Moritz, Esmeralda Garcia,
M. Axel Wollmer, Daniel Umbricht,
Dominique J.F. de Quervain,
Marc Hofmann, Alessia Maddalena,
Andreas Papassotiropoulos, and Roger M. Nitsch*
Division of Psychiatry Research
University of Zurich
August Forel Strasse 1
8008 Zurich
Switzerland

Summary

To test whether antibodies against β-amyloid are effective in slowing progression of Alzheimer's disease, we assessed cognitive functions in 30 patients who received a prime and a booster immunization of aggregated $A\beta_{42}$ over a 1 year period in a placebo-controlled, randomized trial. Twenty patients generated antibodies against β-amyloid, as determined by tissue amyloid plaque immunoreactivity assay. Patients who generated such antibodies showed significantly slower rates of decline of cognitive functions and activities of daily living, as indicated by the Mini Mental State Examination, the Disability Assessment for Dementia, and the Visual Paired Associates Test of delayed recall from the Wechsler Memory Scale, as compared to patients without such antibodies. These beneficial clinical effects were also present in two of three patients who had experienced transient episodes of immunization-related aseptic meningoencephalitis. Our results establish that antibodies against β-amyloid plaques can slow cognitive decline in patients with Alzheimer's disease.

Introduction

β-amyloid is a major histopathological hallmark of Alzheimer's disease (AD) (National Institute on Aging, 1997). It is associated with age-related cognitive decline, neurotoxicity, and the formation of neurofibrillary tangles (NFT) (Naslund et al., 2000; Chen et al., 2000; Geula et al., 1998; Götz et al., 2001; Lewis et al., 2001). Therefore, several β-amyloid-lowering strategies are currently developed for clinical use. These include inhibition of the generation of amyloid β-peptide (Aβ) with β- and γ-secretase inhibitors, prevention of Aβ aggregation, and immunization against β-amyloid (Citron, 2002; Weiner and Selkoe, 2002; Sigurdsson et al., 2002; Gandy, 2002). Both passive and active immunization of transgenic mice against β-amyloid can reverse neuropathology and improve pathologic learning and memory behaviors (Schenk et al., 1999; Bard et al., 2000; Janus et al., 2000; Morgan et al., 2000; De Mattos et al., 2001). In a human patient with AD, immunization against β-amyloid was associated with sizable brain areas devoid of β-amyloid, reduced neuritic pathology, reduced astrocytosis, and microglial cells filled with β-amyloid (Nicoll et al., 2003), as predicted by previous immunization experiments in transgenic mice.

To test whether active immunization can slow the progression of dementia in patients with AD, a recent multicenter study was initiated, but active dosing of the vaccine was suspended after the transient occurrence of clinical signs of aseptic meningoencephalitis in 6% of the cases (Schenk, 2002; Orgogozo et al., 2003). After suspension of dosing, we continued to follow up our cohort of 30 AD patients who participated in this study.

Patients with a clinical diagnosis of mild to moderate AD had received active prime and booster immunizations with preaggregated $A\beta_{42}$(QS-21) (n = 24) or placebo (n = 6) in a double-blind, randomized study design (Hock et al., 2002; Schenk, 2002; Orgogozo et al., 2003). By using a specific and sensitive tissue amyloid plaque immunoreactivity (TAPIR) assay, we observed the sustained generation of antibodies against brain β-amyloid plaques in 20 of our 30 patients (Hock et al., 2002). To determine whether these antibodies were associated with modifications of the clinical course of AD, we tested cognitive functions and capacities of daily living of the patients at baseline (n = 30) and during a 1 year period (n = 28, due to two dropouts).

Results

Human Antibodies Recognized Brain β-Amyloid Plaques

Twenty of thirty patients in this study generated antibodies that specifically recognized β-amyloid plaques on brain tissue sections obtained from transgenic mice expressing in brains both human APP with the Swedish mutation and human presenilin 1 (PS1) with the M146L mutation ($APP^{Sw}xPS1^{M146L}$) (Figure 1; Holcomb et al., 1998). The ten other patients did not generate such antibodies, or had very low serum level at baseline with unchanged levels during the study. Together, this group of patients (n = 9 observed cases at month 12) was used as the control group for comparisons. The presence, or the absence, of the antibodies against β-amyloid was unrelated to the occurrence of aseptic meningoencephalitis in a total of three patients in our cohort. Confocal microscopy images of β-amyloid plaques stained with human immune sera or immune CSF showed close to complete overlap in staining obtained with both the monoclonal antibody 4G8 against Aβ and with Thioflavin S. The overlap in staining with Thioflavin S indicated that these human antibodies recognized bona fide brain β-amyloid plaques. To score the ability of the sera to recognize β-amyloid plaques, we used our TAPIR assay (Hock et al., 2002).

*Correspondence: nitsch@bli.unizh.ch (R.M.N.), chock@bli.unizh.ch (C.H.)

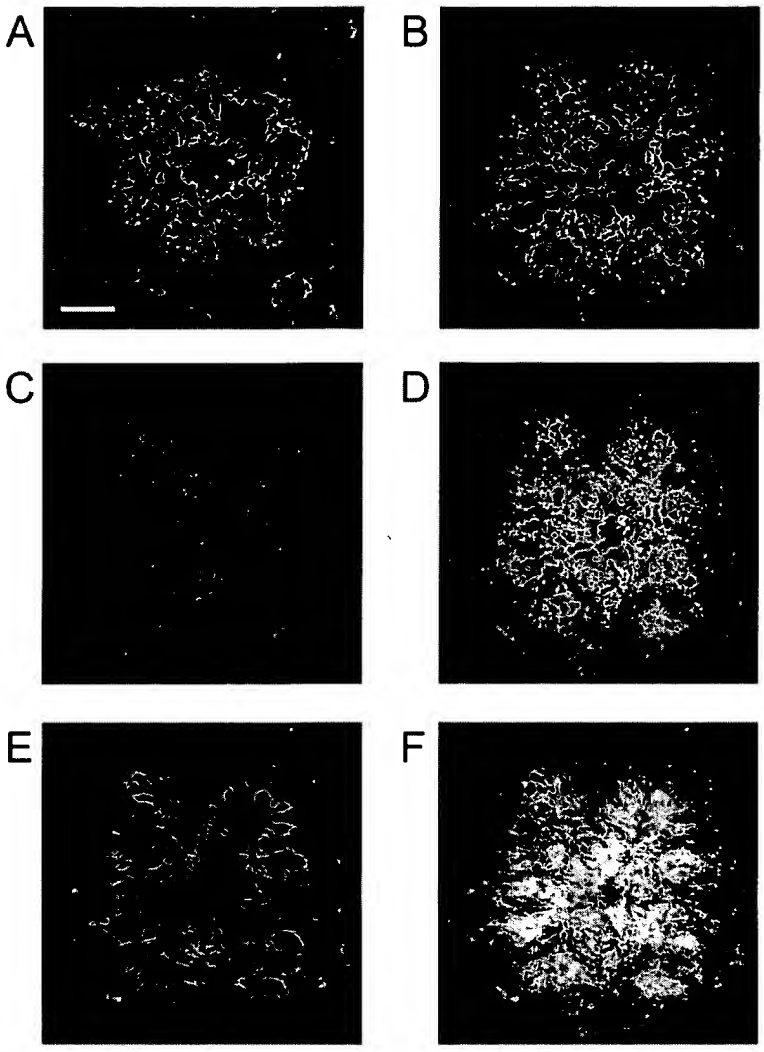

Figure 1. Confocal Immunofluorescence Image of β-Amyloid Plaques Stained by Human Antibodies against β-Amyloid Obtained from a Patient with AD Who Participated in This Study

(A) Human immune serum with antibodies against β-amyloid.
(B–F) Triple-stained β-amyloid plaque.
(B) Human immune CSF, red.
(C) Monoclonal antibody 4G8, blue.
(D) Human immune CSF and 4G8, purple.
(E) Thioflavin S, green.
(F) Human immune CSF and thioflavin S, yellow.
Scale bar equals 20 μm.

Slowed Decline of Cognitive Functions and Capacities of Daily Living

AD patients who generated antibodies against β-amyloid ($n = 19$) performed markedly better on the Mini Mental State Examination (MMSE) 8 months and 1 year after the immunization, as compared to control patients ($n = 9$, $p = 0.008$, ANOVA) (Figure 2A). As compared to baseline, the patients who generated antibodies against β-amyloid remained unchanged after 1 year (-1.4 ± 3.5, mean ± SD n.s., median = -1.0). Patients in the control group worsened significantly by -6.3 ± 4.0 MMSE points (mean ± SD, median = -5.0; $p < 0.01$, Wilcoxon). This magnitude of progression of dementia is clinically relevant, and it is somewhat higher than rates of decline known for the natural history of AD of -3.9 ± 3.7 MMSE points per year, but both our mean and median values are well within one standard deviation of published data (Morris et al., 1993). In contrast, the clinical stabilization in the group of patients who generated antibodies against β-amyloid differed markedly from the known natural history of AD (Morris et al., 1993).

To determine whether beneficial effects were also noted by the patients' caregivers, we applied the Disability Assessment for Dementia (DAD) rating scale by interviewing caregivers in a double-blinded manner (Gauthier et al., 1997). The DAD assesses activities of daily living including initiation, planning, and organization; performance in eating, bathing, grooming, dressing, and toileting; and telephone communication, paying bills, cooking, and shopping. Performance in the DAD was significantly better in patients who generated antibodies against β-amyloid, as compared to control patients (Figure 2B). After 12 months, patients who generated antibodies against β-amyloid declined by -2.9 ± 3.8 of 40 points on the DAD, as compared to -8.7 ± 10.0 in control patients ($p = 0.030$, ANOVA) or, in percent of applicable questions, by $-6.1\% \pm 9.0\%$ versus $-20.1\% \pm 23.4\%$, respectively ($p = 0.039$, ANOVA). Thus, the cognitive stabilization translated into relevance for daily life.

Relation of Clinical Outcome to the Increase in TAPIR Scores

To determine whether the clinical outcome was related to TAPIR scores, we grouped the patients according to the magnitude of increases in TAPIR scores and ob-

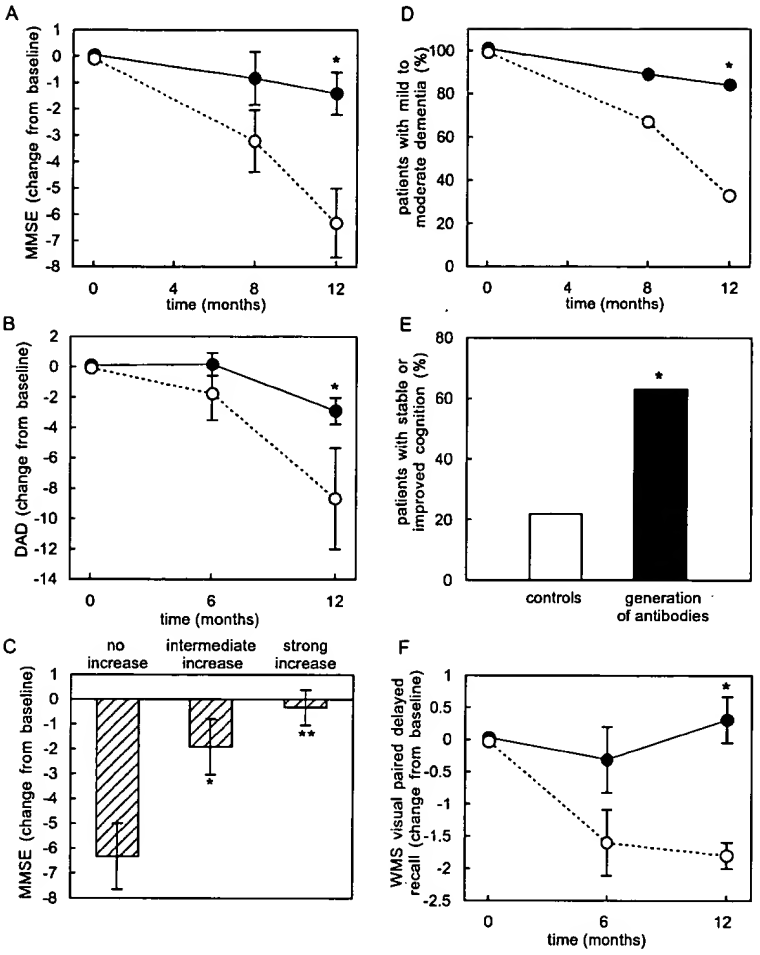

Figure 2. The Generation of Antibodies against β-Amyloid Was Associated with Slowed Declines in Cognitive Functions and Activities of Daily Living

AD patients who generated antibodies against β-amyloid (filled symbols, solid lines) were compared to patients without this immune response (controls, open symbols, dashed lines).

(A) MMSE scores of dementia severity. AD patients who generated antibodies against β-amyloid (n = 19) performed significantly better (*p = 0.008, ANOVA) than the controls (n = 9).

(B) DAD scores of activities of daily living. Patients who generated antibodies against β-amyloid (n = 19) performed significantly better (*p = 0.030, ANOVA) than control patients (n = 9); in percent of applicable questions, patients who generated antibodies against β-amyloid declined by −6.1% ± 9.0% versus −20.1% ± 23.4% decline of controls after 12 months (p = 0.039, ANOVA).

(C) The magnitude of the immune response, as defined by increases in TAPIR scores, was related to the clinical outcome. Whereas control patients without increases in TAPIR scores (n = 9) worsened, patients with intermediate increases (n = 13) declined only marginally, and patients with strong increases (n = 6) remained stable (p = 0.008, Kruskal-Wallis test; *p = 0.021, **p = 0.004, U tests versus controls).

(D) Prevention of disease progression. Upon the generation of antibodies against β-amyloid, significantly more patients did not progress to severe dementia (MMSE < 14). In contrast, the vast majority of control patients had progressed to severe dementia (*p < 0.01, χ^2 = 7.25, d.f. = 1).

(E) Cognitive stabilization. MMSE scores were unchanged (±3) or higher in 12 of 19 patients who generated antibodies against β-amyloid (solid bar) in contrast to 2 of 9 control patients (open bar) (*p < 0.05, χ^2 = 4.09, d.f. = 1).

(F) Preserved hippocampal function tested by the WMS Visual Paired Associates Test of delayed recall. Only two-thirds of the study patients in either group were able to complete this task. Performance of the patients who generated antibodies against β-amyloid (n = 13) was significantly better (*p = 0.029, ANOVA) as compared to control patients (n = 5).

tained the following three groups: no increase (n = 9), intermediate increase (n = 13), and strong increase (n = 6) (Figure 2C). Whereas patients with no increases in TAPIR scores worsened markedly, patients with intermediate increases declined only marginally, and patients with strong increases remained stable (p = 0.008, Kruskal-Wallis test). These data show a dose-response relationship between the increase in serum antibodies against β-amyloid plaques and the clinical outcome. Patients with strong increases in TAPIR scores were essentially protected from disease progression. Notably, two patients in this group improved to MMSE scores of 28 and 30, back from 25 and 24 at baseline, respectively.

Prevention of Disease Progression

At baseline, only patients with mild to moderate dementia (MMSE 16–26) were included in the study. After 1 year, 67% (6 of 9) of the control patients had progressed to severe dementia (MMSE <14). In contrast, only 16% (3 of 19) of patients who generated antibodies against β-amyloid had progressed to severe dementia ($p < 0.01$, $\chi^2 = 7.25$, d.f. = 1; Figure 2D). Moreover, 21% (4 of 19) of patients in this group had improved MMSE scores, as compared to none in the control group. When cognitive stabilization was defined by unchanged (±3 points) or higher MMSE scores, 63% (12 of 19) of the patients who generated antibodies against β-amyloid remained stable, as compared to 22% (2 of 9) in the control group ($p < 0.05$, $\chi^2 = 4.09$, d.f. = 1; Figure 2E).

Preserved Hippocampal Function

Thirteen of twenty of the patients who generated antibodies against β-amyloid and 5 of 10 of the control patients were able to complete the Visual Paired Associates Test of delayed recall from the Wechsler Memory Scale. This task is a demanding test of hippocampal memory function (Wechsler, 1987). The reasons for not completing (n = 12) this test included inabilities to follow instructions or to learn items for recall and refusal. Upon generation of antibodies against β-amyloid, performance of the subset of patients who completed this

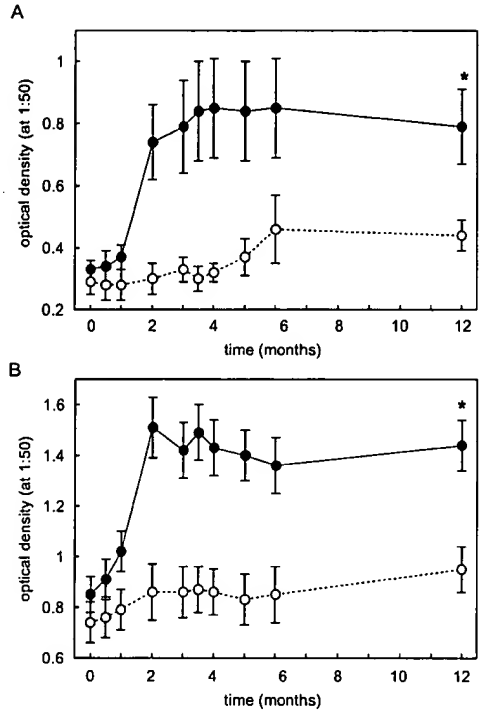

Figure 3. Sustained Increases in Serum Antibodies

Increases in TAPIR scores (n = 20; filled symbols, solid line) were associated with long-lasting increases in serum IgG (A) and IgM (B) antibodies against $A\beta_{42}$ (*p = 0.005 and p < 0.0001, ANOVA). No changes in anti-$A\beta_{42}$-IgG or IgM in the control group (n = 10; open symbols, dashed line).

test was significantly better (p = 0.029, ANOVA), as compared to control patients (Figure 2F).

Other Neuropsychological Tests

The generation of antibodies against β-amyloid was associated with trends toward better test scores in other assessments including the ADAS-Cog (with antibodies: −5.5 ± 6.8, n = 17 versus −7.8 ± 5.2 in controls, n = 6), naming (0.1 ± 0.7, n = 19 versus 0.7 ± 1.0, n = 9), verbal fluency (−3.6 ± 5.1, n = 18 versus −5.9 ± 4.3, n = 8), and CGIC (−0.1 ± 0.7, n = 18 versus −1.1 ± 1.0, n = 8).

Sustained Increases in Serum Antibodies against Aβ

The group of patients who generated antibodies against β-amyloid showed a marked and long-lasting increase in serum antibodies against aggregated $A\beta_{42}$ in both IgG (Figure 3A) and IgM (Figure 3B) classes as measured by ELISA (p = 0.005 and p < 0.0001, ANOVA, two factors, repeated measurements). Titers of both anti-$A\beta_{42}$-IgG and anti-$A\beta_{42}$-IgM increased within the month after the prime injection, attained a maximum within the month after the booster injection, and remained high until month 12. These sustained increases could be related to the long-term stability of the $A\beta_{42}$ aggregates use as the vaccine.

TAPIR Assay Predicts Clinical Outcome

If binding to, and removal of, brain β-amyloid is a therapeutic principle in AD, selective antibodies against β-amyloid should have stronger protective effects than anti-Aβ antibodies without the ability to bind β-amyloid. Indeed, we observed no significant differences in clinical measures, cognitive performance, or neuropsychological tests when ELISA titers of antibodies against Aβ peptides were used to differentiate responders from nonresponders (p > 0.05 in all tests). Moreover, two patients with high ELISA titers but with low TAPIR scores did not experience beneficial clinical effects, but three patients with high TAPIR scores and with low, ELISA titers were protected against disease progression. In addition, there were no stable or improved patients with high ELISA titers and low TAPIR scores, and there were no worsened patients with high TAPIR scores and low ELISA titers (p = 0.025, χ^2 = 5.0; d.f. = 1).

Antibodies against β-Amyloid Can Reach the Brain

We had available 20 paired CSF samples obtained both at baseline and after 1 year. We found that immune CSF of four patients contained antibodies against β-amyloid (Figure 1B), demonstrating the principal ability for the antibodies to reach the CSF compartment. CSF/serum ratios for albumin were normal in the patients with CSF antibodies against β-amyloid; presence of oligoclonal bands in CSF was observed in one patient. Our data favor passage of antibodies across the blood-brain barrier, irrespective of its integrity, over intrathecal production, as an explanation for antibody presence in CSF. The absence of either increased CSF cell counts or increased CSF IgG indices imply that generation of antibodies against β-amyloid is not associated with chronic brain inflammation, although our 1 year CSF data can not rule out transient inflammatory episodes during earlier time points within the study period.

Unchanged Plasma and CSF Levels of Aβ

In our patients, the generation of antibodies against β-amyloid was not associated with major changes in either CSF levels of $A\beta_{40}$ and $A\beta_{42}$ (Figures 4A and 4B) or in plasma levels of $A\beta_{42}$ (Figure 4C), arguing against the possibility that sequestration of serum Aβ is an underlying principle of the therapeutic effects observed here. We do not know whether brain β-amyloid load was reduced in our study patients; in vivo imaging techniques will be required to answer this question.

Discussion

Here we report the results of our TAPIR analysis applied to the Zurich cohort of 30 patients who participated in a multicenter trial of β-amyloid immunization. We observed slowed cognitive decline in AD patients who generated antibodies against β-amyloid plaques. Whereas cognition of patients who did not generate such antibodies worsened, patients with intermediate increases in such antibodies declined only marginally, and patients with strong increases remained clinically and cognitively stable. The clinical stabilization was further substantiated by significantly better performance in activities of

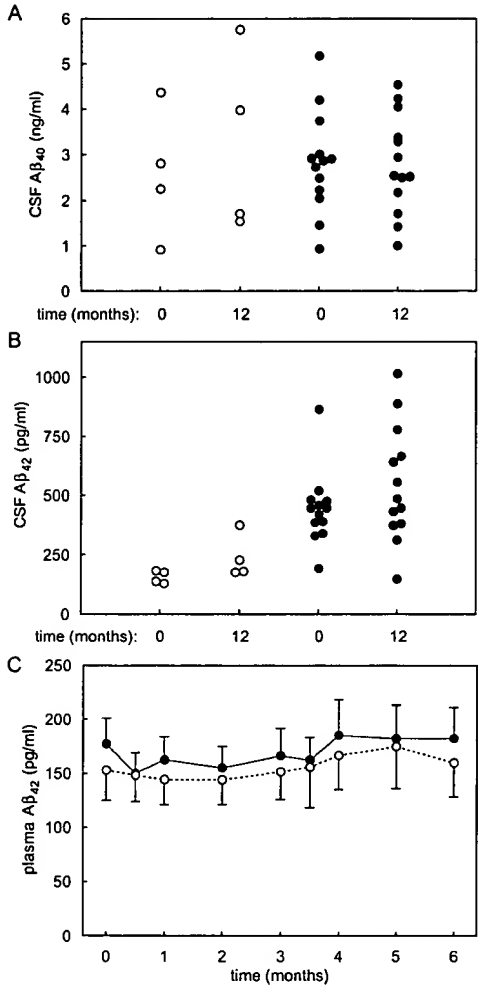

Figure 4. No Differences in CSF or Plasma Levels of Aβ

Patients who generated antibodies against β-amyloid (filled circles) as compared to control patients (open circles).

(A) CSF levels of $A\beta_{40}$.

(B) CSF levels of $A\beta_{42}$.

(C) Plasma levels of $A\beta_{42}$ (means ± SEM, n = 20 patients who generated antibodies against β-amyloid, n = 10 control patients, n.s.).

daily living and by tests of hippocampal memory functions. These data establish the possibility that antibodies against β-amyloid are effective in halting the progression of AD.

Despite the fact that the TAPIR scores were statistically correlated with ELISA titers of serum antibodies against $A\beta_{42}$ (r_s = 0.700, $p < 0.001$), the TAPIR scores predicted therapeutic outcome, whereas ELISA did not. Together with our subgroup of patients with discrepant TAPIR scores and ELISA titers, these data suggest that the degree of selectivity of the antibodies for bona fide β-amyloid is an important determinant for the clinical efficacy of antibodies in AD. The difference between TAPIR and ELISA scores could be related to important qualitative characteristics of the antibodies with respect to epitope recognition, affinity, and avidity of the binding reaction with β-amyloid plaques in the physiologic brain environment. These conditions may not be mimicked adequately by preparations of synthetic Aβ on ELISA plates. Together, the data underscore the importance of using appropriate assays for antibody analyses, and they suggest using TAPIR scores instead of ELISA titers for the analysis of responders. These data also demonstrate the necessity for carefully selecting therapeutically relevant epitopes within β-amyloid and its constituents for the future development of immunotherapy for AD.

The observed clinical differences among AD patients with and without an immune response were unrelated to the AChEI treatments, because patients in both groups were on stable dosages of AChEI before and during this trial. These data therefore support the possibility that the therapeutic effects of antibodies against β-amyloid and AChEI are additive. For the formal test of this possibility, however, control groups without AChEI treatments are required. Other factors that could potentially affect rates of progression of dementia, including age, gender, medication, and head trauma, were either excluded by the selection criteria or were distributed evenly among the groups. The ApoE genotype affects the risk for getting AD as well as the age of onset, but not the rate of cognitive decline once the disease has started (Growdon et al., 1996). Nevertheless, the distribution of the common ApoE genotypes was equal among the groups ($p = 0.114$, $\chi^2 = 2.5$; d.f. = 1 for genotypes, and $p = 0.438$, $\chi^2 = 0.602$; d.f. = 1 for allele frequencies), and there was no carrier of an ApoEε2 allele in our cohort.

During the course of the AN1792 multicenter trial, 6% of the study patients developed clinical signs of aseptic meningoencephalitis (Schenk, 2002; Orgogozo et al., 2003), and they were generally treated with corticosteroids. These signs did not correlate with the generation of antibodies against β-amyloid. Moreover, occurrence of aseptic meningoencephalitis did not predict clinical outcome: two patients with aseptic meningoencephalitis and who generated antibodies against β-amyloid in our cohort remained cognitively stable 1 year after the immunizations, despite the transient and reversible drop during the acute symptoms. On the other hand, dementia severity in one other meningoencephalitis patient without antibodies against β-amyloid continuously declined after recovery from the acute symptoms. These data imply the possibility that the beneficial effects of antibodies against β-amyloid on cognitive functions are maintained even after transient episodes of postvaccination aseptic meningoencephalitis.

Passive immunization of mice with antibodies against soluble Aβ resulted in increased plasma and CSF levels of Aβ within 3 days (Dodart et al., 2002; De Mattos et al., 2002), suggesting that antibody binding to plasma Aβ leads to its sequestration, followed by efflux of Aβ from brain to plasma. Other data show the importance of high-affinity binding of antibodies to F_c receptors for removal of β-amyloid from mouse brain, suggesting F_c receptor-mediated uptake of β-amyloid by macrophages or microglia (Bard et al., 2003). Our data argue against sequestration as an underlying principle of the observed therapeutic effects, but the antibodies against β-amyloid reported here are substantially different than those used in the mouse studies, because of their relative selectivity for structural epitopes in β-amyloid in plaques (Hock et al., 2002).

How do the results of this study affect the status of the amyloid cascade hypothesis of AD? Current versions of this hypothesis claim a primary role of β-amyloid in the pathogenesis of AD (Steiner and Haass, 2000; Selkoe, 2001, 2002; Walter et al., 2001; Hardy and Selkoe 2002; Golde, 2003; Ingelsson and Hyman, 2002; Dominguez and De Strooper, 2002; Sisodia and St. George-Hyslop, 2002). In analogy to infectious disease, where the primary role in causing disease is played by an infectious agent, the characterization of the pathogenic role of β-amyloid can be accomplished by two complementary experiments: transmission and vaccination. Transmission experiments are designed to identify the disease-causing entity in a diseased tissue by isolating the minimal disease-causing entity, transmitting it to a healthy animal, and thereby causing the disease phenotype. For β-amyloid, this was largely accomplished by showing its role in neurofibrillar degeneration and in NFT formation, either by intracerebral microinjection or by transgenic expression (Götz et al., 2001; Lewis et al., 2001). Vaccination provides a complementary approach to explore the role of a suspected disease-causing entity. The experiment uses significant parts of the suspect as a vaccine to induce antibody-mediated immunity in a host animal. If the generated antibodies selectively react with the suspect and protect against disease—after exposure to an otherwise pathogenic dose of the suspected disease-causing entity—a central pathogenic role of the suspect is highly likely. From this point of view, the use of β-amyloid as a vaccine tests the possibility that β-amyloid plays a central role in causing cognitive decline in AD. Our result that precisely the patients who developed antibodies against β-amyloid—but not patients without such antibodies—prevented the progression of AD provides, therefore, the first successful clinical evidence for a central role of β-amyloid in causing cognitive decline and dementia in AD patients. The fact that the degree of the protective effects was related to the magnitude of the immune response against β-amyloid plaques in brain tissue underscores this conclusion.

Important open questions include the relationship of the clinical efficacy to effects of antibodies against β-amyloid on the histopathology of AD. The initial observation of a single immunized case devoid of β-amyloid (Nicoll et al., 2003) is clearly supportive of antibody-mediated removal of β-amyloid, but additional histopathological analyses are required to conclusively confirm that removal of β-amyloid from brain is both necessary and sufficient for clinical efficacy.

In conclusion, our findings establish that antibodies against β-amyloid plaques can slow clinical and cognitive decline in patients with AD, and they indicate major advantages of TAPIR assays over ELISA to predict clinical outcome. Our analyses should be continued by long-term follow-up studies of the complete cohort of AD patients who generated antibodies against β-amyloid as a result of Aβ immunization.

Experimental Procedures

Patients and Treatments

These experiments were done within an additional adjunct study of the Zurich cohort of 30 AD patients (9 female) who participated in the ELAN/Wyeth-Ayerst AN1792(QS-21) Phase 2A multicenter trial prior to unblinding of both treatment status and antibody responses. The study was approved by the ethics committee; written, informed consent was obtained from all patients and caregivers. The clinical diagnosis of probable AD was made according to the NINCDS-ADRDA criteria (McKhann et al., 1984), clinically relevant other diseases were excluded. MRI was done to exclude structural causes of dementia. Patients with mild to moderate dementia (MMSE 21.0 ± 3.2, range 16–26; Folstein et al., 1975) and with disease durations of 3.6 ± 2.3 years (range 1–11) were included. To exclude vascular dementia, Rosen Modified Ischemic scores were <5. The mean age was 72.1 ± 7.2 years (range 52–82). Patients were randomized in a double-blind study design: 24 patients received an active vaccine consisting of preaggregated synthetic $A\beta_{42}$ along with QS-21 adjuvant, and 6 patients received placebo. Both active vaccine and placebo were given as a prime intramuscular injection, followed 1 month later by a boost intramuscular injection. The drug/placebo status remained blinded to patients, caregivers, clinical raters, and laboratory investigators. One patient from the placebo group died during the study from cerebrovascular hemorrhage. One patient refused testing at month 12. Therefore, our study ended with 28 observed cases after one year. The 20 patients (6 female) who generated antibodies against β-amyloid plaques were 73.6 ± 7.0 years old, had baseline MMSE scores of 21.6 ± 3.2, and had a mean duration of disease of 3.6 ± 2.4 years. Nineteen (6 female) of these completed the study (age 73.4 ± 7.1 years, MMSE 21.3 ± 3.1, duration 3.6 ± 2.5 years). The 10 control patients (3 female) were 68.8 ± 6.8 years old, had baseline MMSE scores of 19.9 ± 3.2, and had a mean duration of disease of 3.8 ± 2.3 years. Nine (3 female) of these completed the study (age 68.8 ± 7.2 years, MMSE 19.2 ± 2.5, duration 3.4 ± 2.2 years).

Twenty-eight patients received stable doses of AChEI for at least 3 months prior to inclusion. AChEI were continued throughout the study, except for one patient who generated antibodies against β-amyloid and who terminated AChEI at month 11. The length of treatment with AChEI prior to testing at month 12 was similar among the patient groups with strong increases in TAPIR scores (3.0 ± 2.2 years), with intermediate increases (2.1 ± 0.8), or without increases (3.6 ± 1.9; $p = 0.211$, Kruskal-Wallis test). These time periods are beyond the 1 year period of known stabilizing effects of AChEI (Doody et al., 2001; Giacobini, 2000). Of the patients who generated antibodies against β-amyloid, six received donepezil (5 mg per day, n = 1; 10 mg, n = 5), two rivastigmine (12 mg, n = 1; 3 mg, n = 1), and eleven galantamine (16 mg, n = 5; 24 mg, n = 6). One patient changed from galantamine (16 mg) to rivastigmine (6 mg). Among control patients, five received donepezil (10 mg, n = 5), four galantamine (16 mg, n = 1, 24 mg, n = 3), and one patient no AChEI. Other medications for cognitive enhancement were neither permitted within the trial nor during the 3 month period prior to inclusion. Nonsteroidal antiinflammatory drugs (NSAID), statins, estrogens, and vitamin E were evenly distributed among the two groups. Patients who generated antibodies against β-amyloid used NSAIDs (n = 11), statins (n = 3) vitamin E (n = 2), and no estrogens; patients in the control group used NSAIDs (n = 5), statins (n = 2), vitamin E (n = 1), and estrogens (n = 2).

Tissue Amyloid Plaque Immunoreactivity (TAPIR) Assay

For the assessment of the ability of the human immune sera to react with bona fide β-amyloid plaques in brain tissue, we developed a specific TAPIR assay. Double transgenic mice (18 months) expressing pathogenic AD-causing human mutant APP and PS1 genes ($APP^{sw}xPS1^{M146L}$) were perfused and fixed. Paraffin-embedded brains sections were incubated with human sera or CSF taken at baseline (month 0) and 56.0 ± 5.8 days after the booster injection. Samples were used either undiluted or diluted 1:50 to 1:10,000 in 2% BSA and 10% horse serum in PBS. After washing, human IgG bound to β-amyloid plaques were detected with cy3-conjugated donkey antibodies directed against heavy and light chains of human IgG (Jackson Labs, Bar Harbor, Maine). Fluorescent secondary antibodies were imaged through a 40× objective and a TRITC filter attached to a Nikon Eclipse E800 fluorescence microscope equipped with a Kappa PS 30C CCD camera. Images of all dilutions were acquired with standardized camera settings chosen to be well

below the saturation of 255 arbitrary units (A.U.) in 8 bit mode. The Image J software (http://www.ncbi.nlm.nih.gov) was used to quantify the mean pixel intensities (range 20 to 230 A.U.) of n = 15 β-amyloid plaques per serum dilution. Averages of the means were used for both the standard curve and the individual samples. The assay was linear for serum dilutions ranging from 1:50 to 1:10,000 ($r = 0.951$; $p = 0.013$).

For comparisons with a standard curve obtained by diluting human CSF from a responder, both preimmune and immune sera were used at 1:50 dilutions and categorized by two independent, blinded raters into the following five immunoreactivity scores: absent immunoreactivity (-); weak immunoreactivity corresponding to 1:10,000 (+); moderate, 1:5,000 (++); strong, 1:1,000 (+++); and very strong, 1:500 (++++). To determine the increase in immunoreactivity during treatment, the preimmune immunoreactivity scores were subtracted from the immune scores to generate the following group: no increase, n = 10 (n = 9 observed cases) in the control group. In the group of patients who generated antibodies against β-amyloid (n = 20), one patient dropped out because of unwillingness to participate in neuropsychological testing at month 12, leaving n = 19 observed cases. To compare the degree of the immune response to the clinical outcome, this group was further subdivided into two groups based upon the magnitude of increases in TAPIR scores as follows: strong increases representing 4+ increases from baseline to immune status (n = 6), and moderate increases representing the remaining group of 1+ to 3+ increases (n = 13) from baseline to immune status.

Neuropsychology

Clinical assessments including the MMSE were done at baseline, as well as at month 8 and month 12. Normal MMSE scores were assumed at 27–30; mild dementia at 20–26; moderate at 14–19; and severe at 0–13. The following tests were done at baseline and at months 6 and 12: the Alzheimer's Disease Assessment Scale (ADAS) cognitive part (ADAS-Cog) (Rosen et al., 1984), the Verbal and the Visual Paired Associates Tests of immediate and delayed recall from the Wechsler Memory Scale (WMS) (Wechsler, 1987), and naming and fluency (verbal and categorical) (CERAD) (Morris et al., 1989). Global function was determined by the clinical dementia rating scale (CDRS) (Morris, 1993), as well as the clinical global impression of change (CGIC) (Knopman et al., 1994). Activities of daily living were assessed by Disability Assessment for Dementia (DAD) (Gauthier et al., 1997) rating scale ranging from 0 to 40. Because the Visual Paired Associates Test of delayed recall from the WMS is difficult, several patients were unable to complete it at month 12. The baseline scores for the patients who dropped out of this test were 1.6 ± 1.2 (n = 12), as compared to 2.8 ± 1.5 (n = 18) in patients who completed it after 1 year. The clinical raters remained blinded throughout the study to the treatment status, as well as to the immunoreactivity scores and antibody titers of the patients.

ELISA Titer Assays

Blocked $A\beta_{42}$-coated (Bachem, Weil am Rhein, Germany) microplates (Nunc Maxisorp, Roskilde, Denmark) were incubated with diluted serum samples overnight at 4°C, washed, and incubated individually with goat anti-human biotinylated IgG or IgM (H+L) (Jackson Labs, Bar Harbor, ME), detected by peroxidase-conjugated streptavidin (Jackson Labs, Bar Harbor, ME) and 3,5,3',5'-tetramethylbenzidine (TMB) (Sigma) at 450 nm on a Victor2 Multilabel microplate reader (EG&G Wallac). All samples and standards were assayed in duplicates.

$A\beta_{42}$ and $A\beta_{40}$ ELISAs

CSF and plasma $A\beta_{42}$ were measured by the INNOTEST β-Amyloid1-42 ELISA according to the manufacturer's protocol (Innogenetics, Belgium). For $A\beta_{40}$ ELISA, 1 μg/ml of biotinylated 4G8 (Signet, Dedham, MA) were bound to streptavidin-coated microplates (Nunc) and incubated with CSF diluted in PBS, along with BAP-24 (courtesy Dr. Manfred Brockhaus, Roche), followed by TMB as the chromophor, sulfuric acid and reading at 450 nm. Standard curves of $A\beta_{40}$ (Bachem) scaling from 0.15 to 40 ng/ml were used, and $A\beta_{42}$ was tested as a negative control.

Statistical Analyses

Data were analyzed by ANOVA. Comparisons of two groups were done with Mann-Whitney U tests, and comparisons of three groups were done by Kruskal-Wallis tests. The distribution of categorical variables between groups was tested by using the chi-square and Fisher's exact tests. The correlation coefficient quoted is Spearman's rho. All p values reported are two-sided. Changes in neuropsychological test scores (three data collection time points) were analyzed by observed cases analyses (OC). Changes in serum titers and plasma Aβ levels (ten data collection time points) were analyzed by intention to treat (ITT) analysis; missing values were interpolated between visits and last values were carried forward.

Acknowledgments

The preparations of active vaccine and placebo were administered to the patients within the ELAN/Wyeth-Ayerst AN1792(QS-21) trial. We thank Christin Wilde, Estelle Obrist, Andrea Walter, and Ruth Schmid for excellent clinical study support, Mohamad Jaber for technical support, Drs. Karen Duff and Karen Hsiao-Ashe for transgenic mouse lines, and Dr. Manfred Brockhaus (Roche) for BAP24. We thank Drs. John H. Growdon and Dale Schenk (Elan) for critical comments on this manuscript. This study was funded in parts by the National Centre of Competence in Research on Neural Plasticity and Repair (NCCR), the EU DIADEM program on Diagnosis of Dementia, the Stammbach Foundation, and the University of Zurich.

The authors declare that they have no competing financial interests related to Elan/Wyeth-Ayerst.

Received: March 26, 2003
Revised: April 10, 2003
Accepted: April 30, 2003
Published: May 21, 2003

References

Bard, F., Cannon, C., Barbour, R., Burke, R.L., Games, D., Grajeda, H., Guido, T., Hu, K., Huang, J., Johnson-Wood, K., et al. (2000). Peripherally administered antibodies against amyloid β-peptide enter the central nervous system and reduce pathology in a mouse model of Alzheimer disease. Nat. Med. *6*, 916–919.

Bard, F., Barbour, R., Cannon, C., Carretto, R., Fox, M., Games, D., Guido, T., Hoenow, K., Hu, K., Johnson-Wood, K., et al. (2003). Epitope and isotype specificities of antibodies to β-amyloid peptide for protection against Alzheimer's disease-like neuropathology. Proc. Natl. Acad. Sci. USA *100*, 2023–2028.

Chen, G.Q., Chen, K.S., Knox, J., Inglis, J., Bernard, A., Martin, S.J., Justice, A., McConlogue, L., Games, D., Freedman, S.B., and Morris, R.G. (2000). A learning deficit related to age and β-amyloid plaques in a mouse model of Alzheimer's disease. Nature *408*, 975–979.

Citron, M. (2002). Alzheimer's disease: treatments in discovery and development. Nat. Neurosci. *5*, 1055–1057.

DeMattos, R.B., Bales, K.R., Cummins, D.J., Dodart, J.C., Paul, S.M., and Holtzman, D.M. (2001). Peripheral anti-Aβ antibody alters CNS and plasma Aβ clearance and decreases brain Aβ burden in a mouse model of Alzheimer's disease. Proc. Natl. Acad. Sci. USA *98*, 8850–8855.

DeMattos, R.B., Bales, K.R., Cummins, D.J., Paul, S.M., and Holtzman, D.M. (2002). Brain to plasma amyloid-β efflux: a measure of brain amyloid burden in a mouse model of Alzheimer's disease. Science *295*, 2264–2267.

Dodart, J.C., Bales, K.R., Gannon, K.S., Greene, S.J., DeMattos, R.B., Mathis, C., DeLong, C.A., Wu, S., Wu, X., Holtzman, D.M., and Paul, S.M. (2002). Immunization reverses memory deficits without reducing brain Aβ burden in Alzheimer's disease model. Nat. Neurosci. *5*, 452–457.

Dominguez, D.I., and De Strooper, B. (2002). Novel strategies provide the real test for the amyloid hypothesis of Alzheimer's disease. Trends Pharmacol. Sci. *23*, 324–330.

Doody, R.S., Geldmacher, D.S., Gordon, B., Perdomo, C.A., and Pratt, R.D. (2001). Open-label, multicenter, phase 3 extension study

of the safety and efficacy of donepezil in patients with Alzheimer disease. Arch. Neurol. *58*, 427–433.

Folstein, M.F., Folstein, S.E., and McHugh, P.R. (1975). "Mini-mental state". A practical method for grading the cognitive state of patients for the clinician. J. Psychiatr. Res. *12*, 189–198.

Gandy, S. (2002). Molecular basis for anti-amyloid therapy in the prevention and treatment of Alzheimer's disease. Neurobiol. Aging *23*, 1009–1016.

Gauthier, S., Gelinas, I., and Gauthier, L. (1997). Functional disability in Alzheimer's disease. Int. Psychogeriatr. *9*, 163–165.

Geula, C., Wu, C.K., Saroff, D., Lorenzo, A., Yuan, M., and Yankner, B.A. (1998). Aging renders the brain vulnerable to amyloid beta-protein neurotoxicity. Nat. Med. *4*, 827–831.

Giacobini, E. (2000). Cholinesterase inhibitor therapy stabilizes symptoms of Alzheimer disease. Alzheimer Dis. Assoc. Disord. *14*, S3–S10.

Golde, T.E. (2003). Alzheimer disease therapy: can the amyloid cascade be halted? J. Clin. Invest. *111*, 11–18.

Götz, J., Chen, F., vanDorpe, J., and Nitsch, R.M. (2001). Formation of neurofibrillary tangles in P301L tau transgenic mice induced by Aβ42 fibrils. Science *293*, 1491–1495.

Growdon, J.H., Locascio, J.J., Corkin, S., Gomez-Isla, T., and Hyman, B.T. (1996). Apolipoprotein E genotype does not influence rates of cognitive decline in Alzheimer's disease. Neurology *47*, 444–448.

Hardy, J., and Selkoe, D.J. (2002). The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. Science *297*, 353–356.

Hock, C., Konietzko, U., Papassotiropoulos, A., Wollmer, A., Streffer, J., von Rotz, R.C., Davey, G., Moritz, E., and Nitsch, R.M. (2002). Generation of antibodies specific for β-amyloid by vaccination of patients with Alzheimer disease. Nat. Med. *8*, 1270–1275.

Holcomb, L., Gorson, M.N., McGowan, F., Yu, X., Benkovic, S., Janzen, P., Wright, K., Saad, I., Mueller, R., Morgan, D., et al. (1998). Accelerated Alzheimer-type phenotype in transgenic mice carrying both mutant amyloid precursor protein and presenilin transgenes. Nat. Med. *4*, 97–100.

Ingelsson, M., and Hyman, B.T. (2002). Disordered proteins in dementia. Ann. Med. *34*, 259–271.

Janus, C., Pearson, J., McLaurin, J., Mathews, P.M., Jiang, Y., Schmidt, S.D., Chishti, M.A., Horne, P., Heslin, D., French, J., et al. (2000). Aβ peptide immunization reduces behavioural impairment and plaques in a model of Alzheimer's disease. Nature *408*, 979–982.

Knopman, D.S., Knapp, M.J., Gracon, S.I., and Davis, C.S. (1994). The Clinician Interview-Based Impression (CIBI): a clinician's global change rating scale in Alzheimer's disease. Neurology *44*, 2315–2321.

Lewis, J., Dickson, D.W., Lin, W.L., Chisholm, L., Corral, A., Jones, G., Yen, S.H., Sahara, N., Skipper, L., Yager, D., et al. (2001). Enhanced neurofibrillary degeneration in transgenic mice expressing mutant tau and APP. Science *293*, 1487–1491.

McKhann, G., Drachman, D., Folstein, M., Katzman, R., Price, D., and Stadlan, E.M. (1984). Clinical diagnosis of Alzheimer's disease: report of the NINCDS-ADRDA Work Group under the auspices of Department of Health and Human Services Task Force on Alzheimer's Disease. Neurology *34*, 939–944.

Morgan, D., Diamond, D.M., Gottschall, P.E., Ugen, K.E., Dickey, C., Hardy, J., Duff, K., Jantzen, P., DiCarlo, G., Wilcock, D., et al. (2000). Aβ peptide vaccination prevents memory loss in an animal model of Alzheimer's disease. Nature *408*, 982–985.

Morris, J.C. (1993). The Clinical Dementia Rating (CDR): current version and scoring rules. Neurology *43*, 2412–2414.

Morris, J.C., Heyman, A., Mohs, R.C., Hughes, J.P., van Belle, G., Fillenbaum, G., Mellits, E.D., and Clark, C. (1989). The Consortium to Establish a Registry for Alzheimer's Disease (CERAD). Part I. Clinical and neuropsychological assessment of Alzheimer's disease. Neurology *39*, 1159–1165.

Morris, J.C., Edland, S., Clark, C., Galasko, D., Koss, E., Mohs, R., van Belle, G., Fillenbaum, G., and Heyman, A. (1993). The Consortium to Establish a Registry for Alzheimer's Disease (CERAD). Part IV. Rates of cognitive change in the longitudinal assessment of probable Alzheimer's disease. Neurology *43*, 2457–2465.

Naslund, J., Haroutunian, V., Mohs, R., Davis, K.L., Davies, P., Greengard, P., and Buxbaum, J.D. (2000). Correlation between elevated levels of amyloid beta-peptide in the brain and cognitive decline. JAMA *283*, 1571–1577.

The National Institute on Aging, and Reagan Institute Working Group on Diagnostic Criteria for the Neuropathological Assessment of Alzheimer's Disease (1997). Consensus recommendations for the postmortem diagnosis of Alzheimer's disease. Neurobiol. Aging *18*, S1–S2.

Nicoll, J.A., Wilkinson, D., Holmes, C., Steart, P., Markham, H., and Weller, R.O. (2003). Neuropathology of human Alzheimer disease after immunization with amyloid-beta peptide: a case report. Nat. Med. *9*, 448–452.

Orgogozo, J.-M., Gilman, S., Dartigues, J.-F., Laurent, B., Puel, M., Kirby, L.C., Jouanny, P., Dubois, B., Eisner, L., Flitman, S., et al. (2003). Subacute meningoencephalitis in a subset of patients with AD after Aβ42 immunization. Neurology, in press.

Rosen, W.G., Mohs, R.C., and Davis, K.L. (1984). A new rating scale for Alzheimer's disease. Am. J. Psychiatry *141*, 1356–1364.

Schenk, D. (2002). Amyloid-β immunotherapy for Alzheimer's disease: the end of the beginning. Nat. Rev. Neurosci. *3*, 824–828.

Schenk, D., Barbour, R., Dunn, W., Gordon, G., Grajeda, H., Guido, T., Hu, K., Huang, J., Johnson-Wood, K., Khan, K., et al. (1999). Immunization with amyloid-beta attenuates Alzheimer-disease-like pathology in the PDAPP mouse. Nature *400*, 173–177.

Selkoe, D.J. (2001). Alzheimer's disease: genes, proteins, and therapy. Physiol. Rev. *81*, 741–766.

Selkoe, D.J. (2002). Deciphering the genesis and fate of amyloid beta-protein yields novel therapies for Alzheimer disease. J. Clin. Invest. *110*, 1375–1381.

Sigurdsson, E.M., Wisniewski, T., and Frangione, B. (2002). A safer vaccine for Alzheimer's disease? Neurobiol. Aging *23*, 1001–1008.

Sisodia, S.S., and St. George-Hyslop, P.H. (2002). γ-secretase, notch, Aβ and Alzheimer's disease: where do the presenilins fit in? Nat. Rev. Neurosci. *3*, 281–290.

Steiner, H., and Haass, C. (2000). Intramembraneous proteolysis by presenilins. Nat. Rev. Mol. Cell Biol. *1*, 217–224.

Walter, J., Kaether, C., Steiner, H., and Haass, C. (2001). The cell biology of Alzheimer's disease: uncovering the secrets of secretases. Curr. Opin. Neurobiol. *11*, 585–590.

Wechsler, D. (1987). Wechsler Memory Scale-Revised Manual (San Antonio, TX: The Psychological Corporation).

Weiner, H.L., and Selkoe, D.J. (2002). Inflammation and therapeutic vaccination in CNS diseases. Nature *420*, 879–884.

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 276, No. 24, Issue of June 15, pp. 21562–21570, 2001
Printed in U.S.A.

Early-onset Amyloid Deposition and Cognitive Deficits in Transgenic Mice Expressing a Double Mutant Form of Amyloid Precursor Protein 695*

Received for publication, January 25, 2001, and in revised form, March 9, 2001
Published, JBC Papers in Press, March 15, 2001, DOI 10.1074/jbc. M100710200

M. Azhar Chishti,[a] Dun-Shen Yang,[a] Christopher Janus,[a] Amie L. Phinney,[a,b] Patrick Horne,[a] Jacqueline Pearson,[a] Robert Strome,[a] Noah Zuker,[a] James Loukides,[a] Janet French,[a] Sherry Turner,[c] Gianluca Lozza,[d] Mariagrazia Grilli,[d] Suzanne Kunicki,[e,f] Céline Morissette,[e] Julie Paquette,[e] Francine Gervais,[e], Catherine Bergeron,[a,g,h] Paul E. Fraser,[a,i] George A. Carlson,[c] Peter St. George-Hyslop,[a,j,h] and David Westaway[a,g,k]

From the [a]Centre for Research in Neurodegenerative Diseases, the [g]Department of Laboratory Medicine and Pathobiology, the [i]Department of Medical Biophysics, the [j]Department of Medicine (Division of Neurology), [h]University Health Network, University of Toronto, Toronto, Ontario M5S 3H2, Canada, [c]McLaughlin Research Institute, Great Falls, Montana 59405-4900, [d]Schering-Plough Research Institute, Milan, Italy, and [e]Neurochem Inc., Ville St.-Laurent, Montreal, Quebec H4S 2A1, Canada

We have created early-onset transgenic (Tg) models by exploiting the synergistic effects of familial Alzheimer's disease mutations on amyloid β-peptide (Aβ) biogenesis. TgCRND8 mice encode a double mutant form of amyloid precursor protein 695 (KM670/671NL+V717F) under the control of the PrP gene promoter. Thioflavine S-positive Aβ amyloid deposits are present at 3 months, with dense-cored plaques and neuritic pathology evident from 5 months of age. TgCRND8 mice exhibit 3,200–4,600 pmol of Aβ42 per g brain at age 6 months, with an excess of Aβ42 over Aβ40. High level production of the pathogenic Aβ42 form of Aβ peptide was associated with an early impairment in TgCRND8 mice in acquisition and learning reversal in the reference memory version of the Morris water maze, present by 3 months of age. Notably, learning impairment in young mice was offset by immunization against Aβ42 (Janus, C., Pearson, J., McLaurin, J., Mathews, P. M., Jiang, Y., Schmidt, S. D., Chishti, M. A., Horne, P., Heslin, D., French, J., Mount, H. T. J., Nixon, R. A., Mercken, M., Bergeron, C., Fraser, P. E., St. George-Hyslop, P., and Westaway, D. (2000) *Nature* 408, 979–982). Amyloid deposition in TgCRND8 mice was enhanced by the expression of presenilin 1 transgenes including familial Alzheimer's disease mutations; for mice also expressing a M146L+L286V presenilin 1 transgene, amyloid deposits were apparent by 1 month of age. The Tg mice described here suggest a potential to investigate aspects of Alzheimer's disease pathogenesis, prophylaxis, and therapy within short time frames.

Alzheimer's disease, the most common cause of dementia, has a complex etiology involving both genetic and environmental determinants. It is characterized by cerebral amyloid deposits formed from the amyloid β-peptide (Aβ),[1] neuronal loss, and intracellular deposits denoted neurofibrillary tangles (NFTs), aggregations of hyper-phosphorylated forms of the microtubule-associated protein tau (τ). Genetic analyses of diverse familial Alzheimer's disease (FAD) kindreds indicate biosynthesis of the amyloid β-peptide (Aβ), generated by secretase-mediated endoproteolysis of the amyloid precursor protein (APP), is a common denominator in inherited forms of the disease. In the case of chromosome 21-linked FAD kindreds, mutations in APP are found in close proximity to the endoprotease sites where Aβ is excised by the action of β- and γ-secretases (2–6). Mutations in presenilins 1 and 2 are thought to enhance cleavage of APP at the γ-secretase site (7–9). Finally, the ε4 allele of the ApoE gene, which is correlated with increased susceptibility to late-onset AD (10), is found to enhance the formation of mature plaques in certain APP transgenic mice (11). These genetic data indicate elevated Aβ biogenesis or accumulation is likely a crucial pathogenic event in all forms of AD (*i.e.* both familial and sporadic AD). This conclusion finds a parallel in studies indicating that Aβ is neurotoxic (12–14).

Since there are no naturally occurring rodent forms of AD, there has been great interest in creating accurate transgenic facsimiles of this disease. Such disease models have the potential to stratify pathogenic events and practical utility for testing interventions directed against synthesis or deposition of the Aβ peptide. However, despite intense effort, remarkably few models exist (reviewed in Ref. 15). Some models fail to produce APP and/or its metabolites by physiologically appropriate path-

* This work was supported in part by the Canadian Institutes for Health Research, the Ontario Mental Health Foundation, The Howard Hughes Medical Institute, Natural Sciences and Engineering Research Council of Canada, The Scottish Rite Foundation, the Alzheimer Society of Ontario, the National Institute on Aging, and the Fraternal Order of Eagles. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[b] Supported by Canadian Institutes for Health Research post-doctoral fellowship.

[f] Supported by Natural Sciences and Engineering Research Council of Canada post-doctoral fellowships.

[k] To whom correspondence should be addressed: University of Toronto, Center for Research in Neurodegenerative Diseases, Tanz Neuroscience Bldg., 6 Queen's Park Crescent West, Toronto, Ontario M5S 3H2, Canada. Tel.: 416-978-1556; Fax: 416-978-1878; E-mail: david.westaway@utoronto.ca.

[1] The abbreviations used are: Aβ, amyloid β-peptide; FAD, familial Alzheimer's disease; AD, Alzheimer's disease; ELISA, enzyme-linked immunosorbent assay; APP, amyloid precursor protein; NFTs, neurofibrillary tangles; Tg, transgenic; PS1, presenilin 1; mAb, monoclonal antibody; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; wt, wild type; NSP, non-spatial pre-training; PDAPP, APP transgenic mice constructed using the platelet derived growth factor beta promoter.

ways, and in cases where this caveat does not apply, the transgenic animals may display only facets of the AD phenotype (16). With regard to neuropathology, the phenotypes created thus far include amyloid deposits that closely resemble those seen in AD, selective neuronal loss (in one instance), some hyperphosphorylation of tau, but no deposition of NFTs (17–20). Neuropathological abnormalities in singly transgenic mice may not appear until 6–9 months of age and may not be robust until animals are well in excess of 1 year of age (see "Discussion"). Other complications encountered in these models include hippocampal atrophy (21), neonatal lethality attributed to overexpression of APP (22, 23), and complex and variable relationships between cognitive dysfunction and transgene expression (18, 21, 23–25). Here we describe a new line of transgenic mice that exhibits deposition of Aβ-amyloid and robust cognitive deficits by the age of 3 months. These mice have a demonstrated utility for assessing procedures that interfere with amyloidogenesis (1) and may serve as a platform to create more sophisticated models of AD.

MATERIALS AND METHODS

Construction and Analysis of Tg Mice—The APP695 cDNA cassette was based upon an isolate of the cDNA clone described by Kang *et al.* (26). A *Sma*I to *Spe*I fragment including 90 and 269 base pairs of the wt APP cDNA 5′- and 3′-untranslated region was cloned into the plasmid vector pUC19 (27). This clone was subjected to mutagenesis using the "transformer" protocol (CLONTECH) to convert the 5′ *Sma*I site to a *Sal*I site, with a second *Sal*I site deriving from the pUC19 polylinker. The ~2.4-kilobase pair *Sal*I fragment was excised and inserted into the *Sal*I site of pBR322 (28), to exclude extraneous polylinker sites and thereby facilitate swapping of internal APP restriction fragments containing either Swedish (KM670/671NL) or Swedish plus Indiana (V717F) mutations ("Quick-change", Stratagene Inc.). Completed plasmids were sequenced in their entirety with a total of 12 sequencing primers covering the APP coding region, to exclude the possible presence of erroneous mutations either present in the starting plasmids or introduced during *in vitro* manipulations. *Sal*I fragments of APP or *Xho*I fragments of PS1 (9) were then cloned into cos.Tet (29). *Not*I transgene fragments excised from this cosmid vector were purified and injected into oocytes of different genetic backgrounds as noted, and founder animals were identified by dot-blot hybridization analysis of genomic DNA using a probe within the hamster PrP gene 3′-untranslated region as described previously (9, 30). Double transgenic mice deriving from crosses of transgene heterozygotes were identified by dot-blot hybridization analysis using human APP or PS1 cDNA probe fragments.

Protein Analysis—Western blots were performed by enhanced chemiluminescence as described previously (9), except ECL-Plus (Amersham Pharmacia Biotech) was used in conjunction with a "Storm" imaging system (Molecular Dynamics) for quantitative analyses. For ELISA analysis, C3H/B6 × FVB/N mice at 4, 6, 8, 10, and 26 weeks of age were transcardially perfused with cold saline. The entire brain was removed and snap-frozen until analysis. Cerebral Aβ was solubilized in a 5 M guanidine HCl, 50 mM Tris-HCl, pH 8.0 buffer (31), agitated, aliquoted, and stored at −80 °C. Thawed aliquots were diluted 10-fold or more and assessed for Aβ40 or Aβ42 using commercially available enzyme-linked immunosorbent assays (ELISAs) specific for either Aβ40 or Aβ42 and calibrated with synthetic Aβ peptides (BIOSOURCE International). The Aβ40 ELISA does not display any cross-reactivity with Aβ42 or Aβ43, and the Aβ42 ELISA does not react with either Aβ40 or Aβ43. Each brain was analyzed in duplicate or triplicate, with the average value reported for each brain.

Survival Census—All pups were weaned at an age between 21 and 24 days. The identification of pups genotypes was carried out between 23 and 26 days of age. Therefore, the reliable estimation of Tg mice survival is limited to their post-weaning age. Statistical analysis of survival as a function of genetic background was carried out on three cohorts of TgCRND8 mice as follows: (C57) × (C3H/C57), (FVB) × (C3H/C57), and (C3H/C57/129SvEv/Tac) × (129SvEv/Tac). For comparative purposes the survival of non-Tg littermates was included, and the analysis was performed for the 1st year of life. Since the mortality of non-Tg mice was minimal (1 non-Tg mouse out of the total of 158 included in the analysis died at the age of 26 days), non-Tg mice were pooled across their genetic backgrounds for graphical presentation and statistical analyses. The probability of survival was assessed by the Kaplan-Meier technique (32), computing the probability of survival at every occurrence of death, thus making it particularly suitable for small sample size cases with variable event intervals. The comparisons of cumulative survival curves for each genetic background of mice were performed using Tarone-Ware test, which weighs early events less than log rank or Breslow tests.

Histology and Immunohistochemistry—Mice were anesthetized and perfused with saline in accordance with The Canadian Council for Animal Care guidelines. Generally, brains were removed and bisected in the mid-sagittal plane. One-half was snap-frozen, and the portion for immunohistochemistry was fixed in 10% neutral buffered formalin for a minimum of 48 h. These specimens were then batch-processed on an automatic tissue processor overnight with vacuum on each station to aid penetration. Paraffin sections were cut at 5 microns and affixed to Fisher brand Superfrost/Plus slides to ensure adhesion. Sections were stained by Bielschowsky's silver impregnation, cresyl violet, thioflavine S, and Luxol Fast Blue combined with hematoxylin and eosin, as noted in the figure legends. For general morphological characteristics, 12 TgCRND8 mice from the hybrid C3H/B6 background, age 43–440 days, were studied. Additionally, 5 TgCRND8 mice (n = 2, 213 days; n = 3, 282 days) and 4 non-transgenic controls (n = 3, 213 days; n = 1, 282 days) were sectioned coronally to investigate hippocampal morphology. The percent volume occupied by the dorsal hippocampus within the surrounding brain regions was estimated using the Cavaleri point counting method. Paraffin-embedded brains were serially sectioned on a rotary microtome at a thickness of 10 μm (as described above) and stained with either hematoxylin and eosin or cresyl violet to delineate the hippocampal borders. The hippocampus was defined to include all regions of the hippocampus proper, hilus, and dentate gyrus. To represent the dorsal hippocampal region a total of 6 serial sections were collected for analysis (every 15th section, starting with the first section in which the hippocampus was visible). Brain sections were visualized using a video microscopy system (Zeiss Axoplan, using a lens for × 4 magnification) and a superimposed point grid (680 μm spacing). Points falling over the hemisphere and those falling over the hippocampus were tallied. Volumes were then estimated using the formula: $V = \Sigma_{points}$·area per point·section thickness·section spacing. Student t tests were performed to compare mean volume data.

For immunohistochemistry, all sections were blocked in dilute (3%) hydrogen peroxide and non-immune goat serum. Epitope retrieval, in the form of a 5-min immersion in formic acid, was carried out prior to demonstration of amyloid and synaptophysin immuoreactivity. In all cases the primary antibody was left to react overnight at 4 °C. The remaining steps using the Dako StreptABC complex-horseradish peroxidase-conjugated "Duet" anti-mouse/rabbit antibody kit were completed according to the protocols provided by the manufacturer. End products were visualized with diaminobenzidine. Sections were lightly counterstained with hematoxylin and were resin-mounted. The sources of the antibodies used were as follows: 369 from Sam Gandy; 4G8 *versus* Aβ residues 17–24 from Richard Rubenstein; Aβ42, 3542 from Frédéric Checler; 6F/3D *versus* Aβ residues 8–17, synaptophysin, and anti-ubiquitin from Dako Inc.; 6E10 *versus* Aβ residues 1–16 from Senetek Inc.; glial fibrillary acidic protein monoclonal from Roche Molecular Biochemicals; NF200 from Novo Castra Laboratories; CD11b from Chemicon Labs; and hyperphosphorylated tau, AT8, from Innogenetics, Gent, Belgium. The 6F/3D antibody was of particular use for quantitative studies using image analysis software as it visualized structures (*i.e.* dense-cored plaques) with sharp boundaries.

Behavioral Tests and Data Analysis—Experimentally naive TgCRND8 mice were tested at 11 weeks of age in two cohorts (N_{Tg} = 10, $N_{non\text{-}Tg}$ = 7 in total) in the reference memory version of Morris water maze test. The water maze apparatus, mouse handling, and general testing procedure are described elsewhere (1, 33). Prior to the spatial learning training, all mice underwent non-spatial pre-training (NSP), to assess swimming abilities and familiarize mice with the test (1). Two days following the NSP phase, all mice underwent a reference memory training with a hidden platform placed in the center of one quadrant of the pool (northeast) for 5 days, with 4 trials per day. After the last trial of day 5, the platform was removed from the pool and each mouse received one 60-s swim "probe trial." Escape latency (in seconds), length of swim path (centimeter) and swim speed (cm/s), were recorded using an on-line HVS image video tracking system (33). For the probe trials, an annulus crossing index was calculated, which represents the number of passes over the platform site minus the mean of passes over alternative sites in other quadrants. The index expresses the spatial place preference and controls for alternative search strategies without place preferences, such as circular search (34, 35). Behavioral data was analyzed using a mixed model of factorial analysis of variance. Degrees of

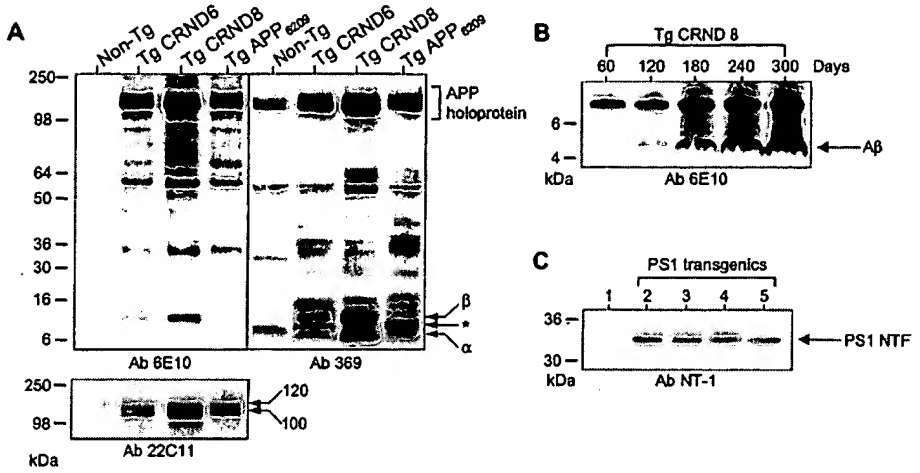

FIG. 1. **Western blot analysis of transgene expression.** 10% brain homogenates made in 0.32 M sucrose were diluted with Laemmli buffer, sonicated, and run on 10–20% Tricine gradient gels (NOVEX). Following transfer to nitrocellulose, human APP and PS1 were detected using C- and N-terminal-specific mAbs and developed by ECL (Amersham Pharmacia Biotech). *A*, comparison of APP expression levels in Tg lines. *1st lane*, Non-Tg; *2nd lane*, Tg CRND6; *3rd lane*, Tg CRND8; and *4th lane*, Tg APP6209. Proteins were detected with human APP-specific C-terminal mAb 6E10 (*left panel*), C-terminal antibody 369 reactive against mouse and human APP (*right panel*), and N-terminal antibody 22C11, also reactive against mouse and human APP (*lower panel*). Positions of high molecular weight APP holoprotein and holoprotein derivatives ("APP holoprotein"), and C-terminal stubs are indicated. *B*, time course of Aβ accumulation in Tg CRND8 mice: *1st lane*, 60 days; *2nd lane*, 120 days; *3rd lane*, 180 days; *4th lane*, 240 days; and *5th lane*, 300 days. Detected with human-specific APP C-terminal mAb 6E10. *C*, comparison of protein expression levels in Tg mice expressing single and double mutants of PS1. Normalized samples of brain homogenates are presented. Detection is with the human-specific PS1 N-terminal mAb NT-1 (72). *Lane 1*, Non-Tg; *lane 2*, TgPS1(L286V)1274; *lane 3*, TgPS1(WT)1098; and *lanes 4* and *5* represent samples from TgPS1(L286V+M146L)6500 mice.

freedom were adjusted by Greenhouse-Geisser epsilon correction for heterogeneity of variance.

RESULTS

Creation of TgCRND8 Mice Expressing Mutant APP

Previous experiments have indicated that overexpression of APP above a threshold of ~4× endogenous is a prerequisite for deposition of amyloid plaques in the central nervous system (18, 22). To avoid the toxic effects associated with these levels of APP overexpression (22, 23, 36, 37), we exploited (i) permissive strain backgrounds and (ii) APP cassettes, including multiple mutations, to maximize production of Aβ for a given level of APP expression. Transgene constructs were based upon a cDNA cassette encoding the major APP isoform in human brain, APP695. This cassette was modified to include either one or two FAD mutations: the "Swedish" mutation (K670N, M671L) and the "Indiana" mutation (V717F), lying adjacent to the N- and C-terminal boundaries of the APP Aβ domain, respectively. APPSwe and APPSwe+717 cDNAs were introduced into cos.Tet (29), a cosmid-based expression vector derived from the Syrian hamster prion protein gene. This vector directs position-independent transgene expression in central nervous system neurons and, to a much lesser extent, astrocytes (38–41). Microinjections into C3H/HeJ × C57BL/6J or (C3H/HeJ × C57BL/6J) × C57BL/6J oocytes (the strains are hereafter referred to as C57 and C3H) yielded a number of putative founders but just two stable transgenic lines designated Tg CRND6 and TgCRND8. These lines harbor APPSwe and APPSwe+V717F transgenes, respectively.

Expression of APP and Aβ Peptide in TgCRND8 Mice

APP-specific antibodies were used to establish transgene expression from founder lines, with previously characterized TgAPPwt6209 transgenic mice providing a point of reference (22). Use of the N-terminal antibody 22C11, which reacts with mouse and human APP, demonstrated overexpression of the full-length mature form of APP of ~120 kDa and different lower molecular mass species of 100 kDa (which are not resolved in this gel system), including immature APP, and APP cleaved at the α- and β-secretase sites, $APP_{S\alpha}$ and $APP_{S\beta}$ (Fig. 1*A*). Overexpression in the TgCRND8 line relative to mouse APP holoprotein detected in non-Tg controls was estimated by quantitative image analysis at ~5-fold. Similar results for high molecular weight APP species were obtained with antibody 369, which reacts with an epitope close to the C terminus of APP shared by mouse and human APP (Fig. 1*A*). Lower molecular weight species deriving from APP processing were also observed in brain extracts of TgCRND8 and TgCRND6 mice analyzed with the human APP-specific monoclonal antibody 6E10 antiserum (epitope positioned N-terminal to the α-secretase cleavage site) and antibody 369. These polypeptides represent APP C-terminal stubs arising from cleavage at the α- and β-secretase sites, with antibody 369 recognizing both species and antibody 6E10 recognizing only the longer β-stubs. As anticipated, the APP6209 Tg line encoding wt human APP does not exhibit β-stubs (as it lacks the Swedish mutation that favors cleavage at this position) but exhibits α-stubs with a reduced electrophoretic mobility due to the inclusion of a c-Myc epitope tag within the C terminus of the APP cDNA cassette (22).

In TgCRND8 mice, increasing levels of a 4-kDa species (but not β-stubs) were detected by Western blot analysis as the animals aged (Fig. 1*B*). To investigate the composition of these 4-kDa Aβ peptide species, we performed ELISAs specific for Aβ40 and Aβ42. Both human Aβ40 and Aβ42 were detected in the brains of Tg CRND8 mice. No signals above background were detected in non-Tg animals. Levels of both peptides increased with age, although in different fashions (Table I). Thus Aβ40 levels were stable between 4 and 10 weeks of age. Aβ42 increased slowly between 4 and 8 weeks, with a potent increase at age 10 weeks, such that it predominated over Aβ40 by a ratio of ~5:1. There was considerable spread in Aβ40 and Aβ42 levels in 10-week-old mice, with levels of Aβ40 varying from 25 to 234 ng/g of brain and Aβ42 ranging from 115 to 728 ng/g of brain. The increase in Aβ42 and the sample-to-sample variation between mice at age 10 weeks likely represents a transition point as Aβ42, first present in soluble form, begins to assemble into insoluble amyloid deposits. Measured at 6 months of age, levels of both Aβ42 and Aβ40 were enormously

TABLE I
Aβ peptide species in young TgCRND8 mice

Age	Gender[a]	Aβ42[b]		Aβ40[b]		Aβ42/Aβ40
		Mean ± S.D.	Range	Mean ± S.D.	Range	Mean ± S.D.
weeks						
4	3F, 3M	40.9 ± 4.1	35–46	55.2 ± 3.6	49–60	0.74 ± 0.04
6	4F, 3M	55.3 ± 6.9	47–63	61.3 ± 11.4	48–82	0.93 ± 0.19
8	4F, 3M	96.9 ± 56.3	59–215	55.8 ± 4.8	46–63	1.07 ± 0.83
10	9F, 1M	298.1 ± 209.9	115–728	69.5 ± 61.1	25–234	5.06 ± 1.56
26	5M	20,783 ± 6599	12,476–29,260	10,584 ± 1495	9,262–13,157	1.96 ± 0.59

[a] Using unpaired *t* tests, the levels of Aβ40 and Aβ42 were not found to differ between males and females, from 4 to 8 weeks of age.
[b] Values are expressed as nanograms of peptide per g of brain (wet weight) derived from duplicate or triplicate determinations of each animal.

increased and were ~510 and 190 times, respectively, the levels observed in 4-week-old mice (which are free of amyloid plaque deposits; Fig. 5*A*).

Postnatal Lethality in TgCRND8 Mice

To gain insight into the ability of genetic backgrounds to modulate lethality associated with APP overexpression (and, from a practical point of view, to preempt premature extinction), the newly established TgCRND8 line was bred to different strain backgrounds. Progeny of an F1 cross to the C3H/HeJ ("C3H") strain were bred to mice derived from FVB/N and 129SvEv/Tac backgrounds. Estimated Kaplan-Meier cumulative survival curves for TgCRND8 mice and their littermates during post-natal development are presented in Fig. 2. Inspection of the curves clearly indicates improved survival of mice with the APP transgene expressed on the (C57) × (C3H/C57) genetic background. In this cohort of Tg mice (n = 52), 20 mice died before the age of 120 days, decreasing their survival to 60% and with three deaths at 130 days and three further deaths after 250 days. When the APP transgene was expressed on either (C3H/C57/129SvEv/Tac) × (129SvEv/Tac) or (FVB) × (C3H/C57) genetic backgrounds (n = 12 and n = 41 respectively), survival dropped rapidly to 25–40% within the first 120 days of their post-natal life (Fig. 2). After this time point, survival with the (C3H/C57/129SvEv/Tac) × (129SvEv/Tac) genetic background dropped slightly to 33% (one death at 159 days) with only 25% (3 mice) of the cohort surviving until 365 days. Similarly, the survival of the TgCRND8 mice with (FVB)×(C3H/C57) background dropped rapidly within the first 120 days of post-natal age (Fig. 2) with 17% of mice (n = 7) surviving until 365 days of age. The survival of mice with the (C57) × (C3H/C57) was significantly better than survival of Tg mice with (FVB) × (C3H/C57) or (C3H/C57/129SvEv/Tac) × (129SvEv/Tac) backgrounds (Tarone-Ware statistics: 5.13, $p < 0.05$, and 19.01, $p < 0.001$, respectively). The survival curves of the latter two genetic backgrounds did not differ significantly from each other (Tarone-Ware statistics: 0.42, $p > 0.05$), and the survival of TgCRND8 mice with the three genetic backgrounds was significantly different from the survival of non-Tg littermates (Tarone-Ware statistics >50, all p values < 0.001.

Although these data suggest significantly increased mortality of TgCRND8 mice as a consequence of a genetic contribution of 129SvEv/Tac or FVB mouse strains, some caveats have to be taken into consideration. First, the relatively small sample sizes of the studied cohorts, especially with the 129SvEv/Tac strain, where only a few mice survived for a long period, may not reliably reflect survival rates at later stages of life. The comparisons of larger cohorts should provide better estimation of survival curves. Second, future survival censuses must be extended to the pre-weaning developmental stage. The selective survival of pups before weaning, or for that matter at the pre-natal stage of development, may cause a bias of a particular cohort entering post-weaning stage. Also, the genetic composition of the particular outbred TgCRND8 parent might be a greater contributor to the differences in survival among the crosses than the composition of the inbred parent. Nonetheless, the major finding of the survival analysis, that the (C57) × (C3H/C57) genetic background significantly reduces mortality in TgCRND8 mice, is in accord with our starting hypothesis. Fifty percent of the studied cohort of 52 mice survived for a year with minimal mortality observed after the first 3 months of age. As shown in previous investigations, the cause of post-natal lethality was not obvious (22). No overt changes were revealed by routine histopathology, although it should be noted that seizures were observed in a small fraction of TgCRND8 animals, and APP transgenes have been correlated with altered vascular responses (37).

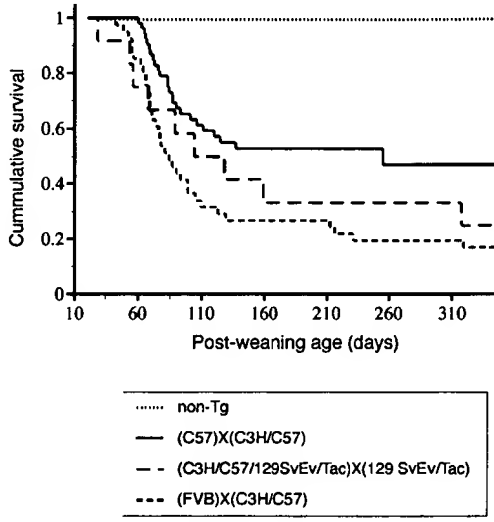

FIG. 2. **Cumulative survival curves (Kaplan-Meier survival analysis) of TgCRND8 mice in different genetic backgrounds.** Cumulative survival curves of TgCRND8 mice as a function of the transgene genetic background. The survival of Tg mice on the (C57) × (C3H/C57) genetic background (n = 52) was the highest, with 50% of mice surviving until 365 days of age (upper cut-off of the analysis). In contrast, the survival of the Tg mice on (C3H/C57/129SvEv/Tac) × (129SvEv/Tac) and (FVB)×(C3H/C57) backgrounds (n = 12 and n = 41 respectively) was affected by increased mortality within the first 120 days of their post-natal age. In the case of Tg mice with the (C3H/C57/129SvEv/Tac) × (129SvEv/Tac) genetic background, only 3 (25%) out of 12 mice survived until the age of 365 days, and only 17% (7 out of 41) of the (FVB) × (C3H/C57) Tg mice reached the age of 365 days.

Cognitive Changes in TgCRND8 Mice

Non-spatial Pre-training—Partial results related to the impaired acquisition of spatial information, as measured by the escape latency, were reported previously for a small cohort of mice (1). Here we present a characterization of a larger cohort of mice, and we include their behavioral analysis during NSP. The analysis showed that during NSP at age 10.5 weeks TgCRND8 mice performed comparably to non-Tg littermates when randomly searching the pool for a hidden platform. Escape latencies and lengths of search paths in the last trial of

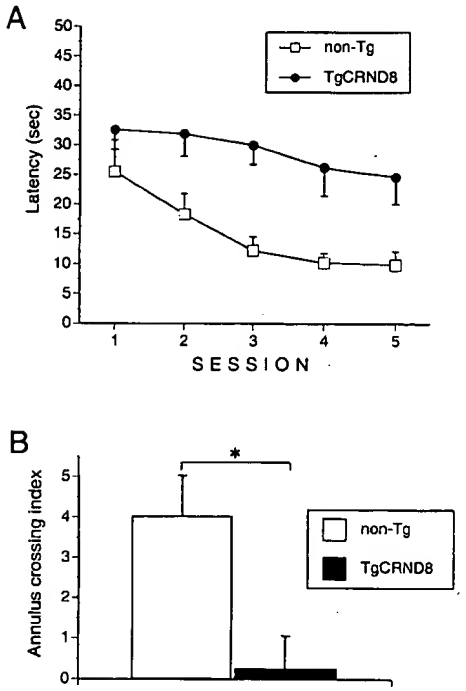

FIG. 3. **Reference memory version of Morris water maze test in TgCRND8 mice.** *A*, at 11 weeks of age, experimentally naive TgCRND8 mice (N_{Tg} = 10) show significant impairment in the acquisition of the spatial information relative to their non-Tg littermates ($N_{non\text{-}Tg}$ = 7). *B*, represents an annulus crossing index (the number of passes over the platform site in TQ, minus the mean of passes over alternative sites in other quadrants) during the probe trial administered after the last training trial of day 5. A positive index indicates selective focal search of the previous platform position, an index approximating zero reflects non-spatial or circular search of the pool. The TgCRND8 mice showed a significantly impaired ($p < 0.01$) spatial bias for the platform position as compared with their non-Tg littermates. *, $p < 0.01$. *Vertical bars* represent S.E.

NSP for the groups were not significantly different (50.0 ± 7.3 *versus* 57.6 ± 8.8 s for latency and 961.1 ± 165.0 *versus* 1351 ± 247.4 cm for path-length, for the non-Tg and Tg mice, respectively). A "visible platform trial" administered during NSP, where the position of the submerged platform was marked by a striped beacon, also failed to reveal differences in performance between non-Tg and Tg groups. Average latencies to reach the cued platform were 9.9 ± 2.0 and 9.1 ± 1.6 s for non-Tg and Tg mice, respectively. The swim paths were 167.3 ± 16.1 cm for non-Tg and 153.1 ± 14.2 cm for Tg mice, and both groups had comparable swim speeds of 21.6 ± 1.4 and 20.2 ± 1.7 cm/s for non-Tg and Tg mice, respectively. In conclusion, these analyses showed TgCRND8 mice performed a random search comparable to non-Tg littermates when presented with the submerged platform and had similar swim paths to a visible platform when extra-maze distal spatial cues were occluded by a curtain.

Water Maze, Reference Memory Test—TgCRND8 mice at 11 weeks showed impairment in the acquisition of spatial information during place discrimination training. They had significantly longer escape latencies to reach the escape platform (Fig. 3*A*; $F(1,15) = 17.98$, $p < 0.001$) and longer search paths ($F(1,15) = 15.91$, $p < 0.001$). Both, Tg and non-Tg groups significantly improved during training ($F(4,60) = 3.29$, $p < 0.02$; $F(4,60) = 3.33$, $p < 0.02$, the latency and path, respectively), and no significant interaction between the groups and sessions was found in both measures. The concordance between measures of latency and search path is not surprising, because the TgCRND8 mice did not differ significantly from the non-Tg littermates in their swim speed during the test ($F(1,15) = 2.53$, $p > 0.05$). The pronounced spatial learning impairment of Tg mice was confirmed during the probe trial administered after the completion of training. They showed lower ($t(15) = 2.99$, $p = 0.01$) annulus crossing index (Fig. 3*B*), searching the pool in a circular fashion and frequently crossing the centers of alternative quadrants (with an annulus crossing index approaching a zero value).

Neuropathology in TgCRND8 Mice

TgCRND6 mice expressing the Swedish mutant form of APP (see Fig. 1) on a B6 × C3H background failed to exhibit cerebral amyloid at ages up to 450 days. Similar results were obtained for two other Tg lines resulting from the microinjection of the same DNA construct into a FVB/N × 129SvEv/Tac background (not shown). On the other hand, hemizygous TgCRND8 mice expressing the double mutant form of APP exhibited potent deposition of cerebral amyloid, present in all animals by 90 days of age. In contrast to PDAPP mice expressing a V717F APP transgene (21), no difference in the volume of the dorsal hippocampus (or in the surrounding regions) was detected between Tg and non-Tg mice ($p < 0.05$ for all values). In TgCRND8 mice, the dorsal hippocampus occupied ~11.0 ± 0.3% of the volume of coronal sections (means ± S.E. of the mean), whereas in the non-Tg mice this value was 10.3 ± 0.2%. Also, the neuronal cytoarchitecture of the TgCRND8 mice appeared normal.

Amyloid deposits in TgCRND8 mice were successfully stained with Aβ42-specific antibodies, as anticipated from prior studies of the mechanism of action of the V717F mutation (5). In addition to confirming dense-cored deposits in aged TgCRND8 mice, the human APP-specific monoclonal antibody 4G8, and to a lesser extent monoclonal antibody 6F/3D, also detected diffuse immuno-staining in the neuropil (using formic acid-treated sections: Fig. 4*B*). These findings were compatible with both the previously described specificity of the 4G8 antibody (42, 43), and the propensity of other mice expressing APP codon 717 mutations to generate "diffuse" amyloid deposits (19, 21, 23). Although human APP was also expressed systemically in TgCRND8 mice, in accord with the tropism of the PrP gene promoter (44), amyloid deposits were not apparent by immunostaining in the kidney, lung, skeletal, and cardiac muscle of aged animals (not shown). These observations suggest a role for tissue-specific factors affecting amyloid deposition and/or APP processing.

Plaque Ontogeny—A single plaque was noted in one TgCRND8 animal at 43 days. Plaque load increased with age and spread to more regions of the brain such that multiple plaque deposits were present per sagittal section in most mice at 65 days of age, and in all mice by 90 days of age. Although studies on large cohorts of mice are in progress, analyses of representative mice revealed between 11–35 and 22–43 plaques in a mid-sagittal section of the cortex at the ages of 90 and 150 days, whereas burdens were greatly increased at 240 and 350 days of age (127–416 and 528–1024 plaques, respectively). A similar pattern of increase pertained to the hippocampal formation with 1–5 and 11–12 plaques at 90 and 150 days and 39–65 and 123–147 plaques at 240 and 360 days, respectively. Thus, the time period for a rapid increase in amyloid burden assessed histologically lags slightly behind that determined biochemically by ELISA assays (Table I).

Plaque Distribution—The plaque present at 43 days was located in the subiculum. The frontal cortex of TgCRND8 mice often had several plaques by 65 days, whereas plaques were rare or absent in the white matter tracts (corpus callosum and alveus) and CA1 region of the hippocampus at this time. By 101 days plaques were widespread in many regions of the cortex as well as in the hippocampus proper, the dentate gyrus, the

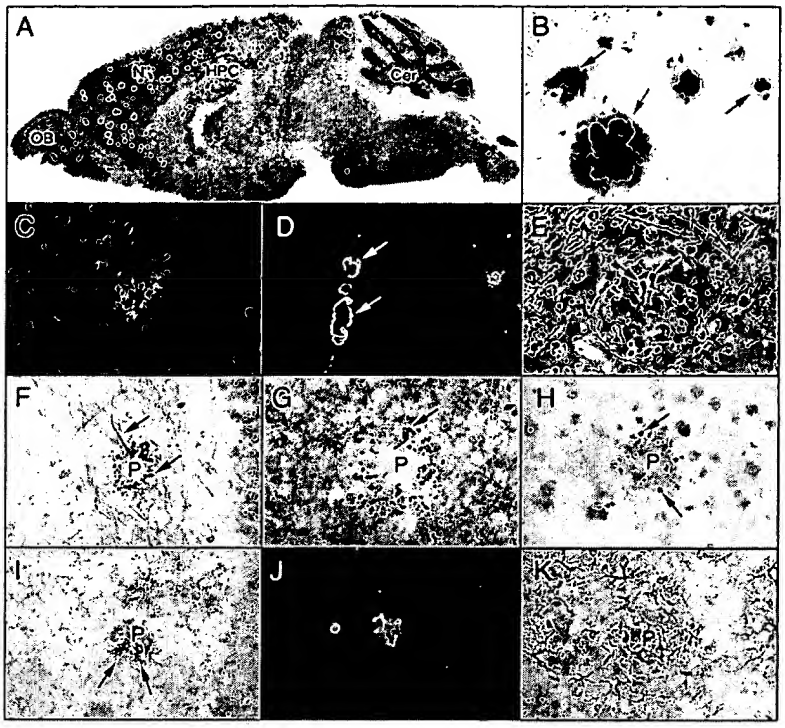

FIG. 4. **Properties of amyloid deposits from TgCRND8 mice.** *A*, sagittal section of an 8-month-old TgCRND8 mouse immunostained with 4G8 anti-Aβ antibody: *Cer*, cerebellum; *HPC*, hippocampus; *N*, neocortex; *OB*, olfactory bulb. *B*, dentate gyrus (× 400 magnification) in a 12-month-old TgCRND8 mouse immunostained with 6F/3D antibody and illustrating different varieties of amyloid deposits: dense-cored (*red arrow*), multicored (*gray arrow*), and diffuse (*black arrow*). *C–K* illustrate the tinctorial and immunostaining profiles of amyloid deposits in a 5.5-month-old TgCRND8 mice (× 400 magnification). *C*, staining with Congo Red showed typical *apple green* and *orange* birefringence with a polarized light source. *D*, perivascular amyloid stained with thioflavine S (*white arrows*). Bielschowsky stain (*E*) and NF-200 (*F*) reveal dystrophic neurites (*arrows*) adjacent to plaques (*P*). *G* and *H* represent synaptophysin and ubiquitin-positive structures in the periphery of plaque deposits, indicative of dystrophic buttons. *I–K* illustrate focal inflammatory responses. *I*, anti-CD11b antibody, *J*, thioflavine S stain of an adjacent section, and *K*, GFAP staining of a third adjacent section.

olfactory bulb, and within the pial vessels. The thalamus (111 days), then the cerebral vasculature and striatum (196 days), followed by the cerebellum and brain stem (243 days) all became progressively burdened by plaques. This pattern of deposition, with cortex and hippocampus affected early on and the cerebellum spared until a late stage in disease, is similar to that seen in AD (45).

Plaque Morphology—The first plaques observed were small, cored deposits, with no radiating amyloid surrounding them (65 days). By 101 days, the plaques varied in size, with some larger plaques having haloes of radiating amyloid. By 131 days the plaques became more heterogeneous in nature. Plaques varied greatly in size as the age of the mice increased, with some being multicored in older animals. Diffuse amyloid deposits appeared in the olfactory bulb at an early stage (101 days). However, outside of the olfactory bulb, diffuse amyloid (*i.e.* amyloid not obviously associated with a cored plaque) did not appear until 243 days, as detected with the 6F/3D antibody. Diffuse amyloid was found primarily in the caudate, cerebellum, and molecular layer of the dentate gyrus (all at 243 days). By 315 days diffuse amyloid appeared throughout the cortex (see Fig. 4*B*).

The majority of amyloid plaque deposits in TgCRND8 mice, including those first to appear at 65 days, stained positive for thioflavine S. These early deposits also revealed Congo Red birefringence. Together these data indicate the deposited amyloid peptide adopts a β-sheet conformation. Amyloid plaques in TgCRND8 mice were associated with dystrophic neurites, as indicated by a variety of histochemical and immunohistochemical stains (Fig. 4). For example, Bielschowsky silver impregnation revealed dystrophic neuritic processes around plaque cores (Fig. 4*E*). Similar structures were imaged by antibodies raised against the 200-kDa isoform of neurofilament (NF-200; Fig. 4*F*), synaptophysin (Fig. 4*G*), and ubiquitin (Fig. 4*H*). The first enlarged plaque-associated neurites were seen with Bielschowsky, synaptophysin, and NF-200 staining at 111 days of age. Dystrophic pathology became more evident as the mice aged further and the frequency of large, dense-cored plaques increased. Finally, it was noteworthy that dystrophic neurites were only observed in the immediate vicinity of plaques, indicating these structures are a direct consequence of amyloid deposition in the TgCRND8 mice.

Neuroinflammation—Mature plaques in TgCRND8 mice were associated with a focal inflammatory response. Elongated cells in periphery of dense-cored amyloid deposits visualized by Luxol fast blue staining or focal staining with a *Griffonia simplicifolia* lectin I isolectin B4 stain (not presented) and staining with anti-CD11b antibody probe (Fig. 4*I*) were consistent with the presence of microglial cells. This microglial activation was accompanied by intense local astrocytic gliosis, illustrated by GFAP staining of adjacent sections encompassing a thioflavin-positive plaque (Fig. 4, *K* and *J*). This astrocytic response clearly exceeded a low basal level of staining of GFAP-positive astrocytes (mostly evident within white matter tracts) noted in both transgenic and non-transgenic mice.

Acceleration of Amyloid Deposition by Co-expression of Presenilin-1 Transgenes

Amyloid deposition in TgCRND8 mice was enhanced by mutant human PS1 transgenes co-expressed with human APP via usage of the same cos.Tet transgene vector (Fig. 5, *right-hand panels*). This effect was evident in terms of a potent increment in plaque burden over age-matched TgCRND8 single transgenic controls. Conversely, expression of wt human PS1 had no overt effect upon amyloid burden (not shown). With a PS1 transgene incorporating two FAD mutations in *cis* (M146L and L286V (46)), the potentiation was particularly remarkable. Here there was robust deposition of plaques by 30–45 days of age (33 days of age presented in Fig. 5*B*). Notably, the graded effects observed for wild type, single, and double mutant transgenes upon amyloid deposition cannot be attributed to different PS1 expression levels, as these were very closely matched between the three selected TgPS1 lines (wild type, mutant, and double mutant, Fig. 1*C*).

DISCUSSION

Factors Affecting APP Transgenesis—Although transgenesis is generally regarded as a routine technique, the APP gene,

first cloned over a decade ago, can be seen to present particular challenges. Difficulties encountered thus far include low expression levels in first generation Tg mice, neonatal lethality associated with high level expression in second generation mice, and physiological endoproteolysis to generate multiple subfragments with diverse biological activities, some of which (*e.g.* neuroprotection (reviewed in Ref. 47) may confound the study of neuropathogenesis. We modified a number of parameters in our experimental design in an effort to reduce the "noise" from these confounding effects and thereby facilitate study of the pathogenic attributes of the Aβ peptide; such pathogenic attributes are clearly suggested by genetic, neuropathological, and toxicological studies (12–14, 48). Our strategy involved using the following: (i) an expression cassette that retained ~90 nucleotides of the APP mRNA 5′-untranslated region adjacent to the start codon, as APP is expressed at high levels for a single copy gene and likely already contains optimized translational initiation signals; (ii) a prion protein cosmid vector that can drive high level pan-neuronal expression in the central nervous system; (iii) two pro-endoproteolytic FAD mutations, to obtain a high level of Aβ peptide for a given level of APP expression; and (iv) use of a genetic background that may offer a degree of protection against high level APP expression favored by parameters i and ii.

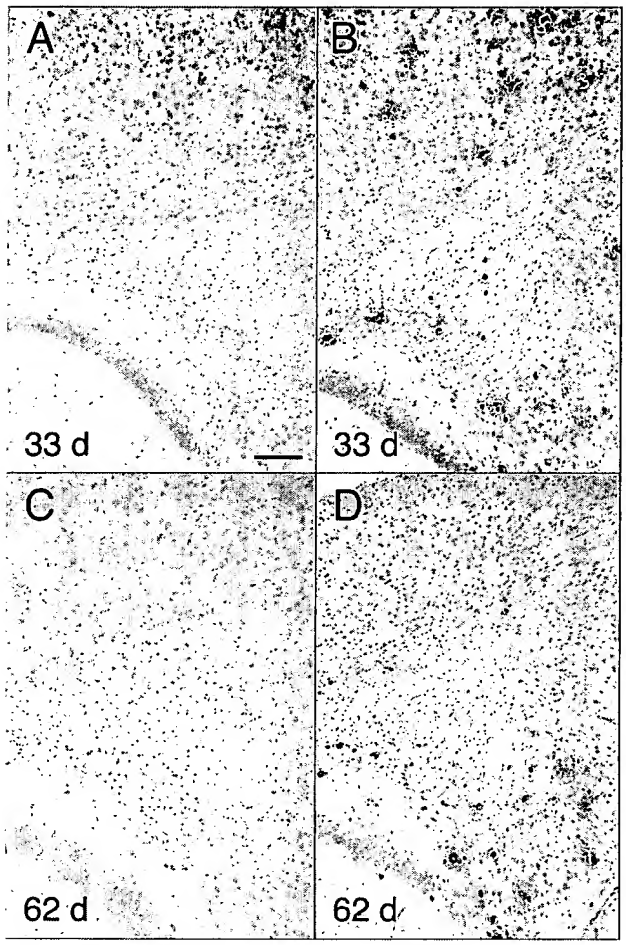

FIG. 5. **Aβ-containing plaques in the cortex of single and double transgenic mice.** Panels show sagittal sections of the neocortex adjacent to the hippocampus. *Left-hand panels* (*A* and *C*) depict "single" transgenic TgCRND8 mice. *Right-hand panels* (*B* and *D*) depict "double" transgenic TgCRND8 mice co-expressing either human presenilin 1 (*B*, TgPS1(M146L+L286V)6500; *D*, TgPS1(L286V)1274). Each *horizontal row* represents single and double -Tg littermates at the same age: *A* and *B*, 33 days; *C* and *D*, 62 days. Immunohistochemical analyses presented were obtained with the 4G8 monoclonal antibody. Note enhanced plaque deposition in double Tg animals. × 400 magnification.

It is possible that genetic background may have been important for establishing the TgCRND8 line. Our strategy was based upon the hypothesis that dominant alleles protective against APP overexpression present in the C3H background (36) would facilitate the establishment of Tg lines with high levels of expression. As injections into C3H oocytes are not usually attempted, the TgCRND8 line was established in an outbred C3H/B6 background. Survival curves presented here document that in TgCRND8 mice high levels of Aβ peptide and modest levels of APP α- and β-stubs can be tolerated in a C3H/B6 genetic background without grossly compromising viability (Fig. 2). Whereas these data are in accord with the starting hypothesis, they are incompatible with facile genetic analysis, as confounding effects due to independent segregation of alleles from the C3H and B6 strains conferring protection or sensitivity to APP overexpression cannot be excluded. Experiments to establish C3H congenic lines of TgCRND8 mice will lead to more definitive information on this point. Furthermore, it is of interest to note that the KM670/NL671+V717F construct injected into oocytes from another potentially protective genetic background (129SvEvTac × FVB/N (36)) yielded a transgenic line designated Tg19959, which also exhibits cerebral plaques at 3 months of age (data not shown). In sum, our results suggest manipulation of genetic background may reduce postnatal death associated with APP overexpression and should be considered in strategies for APP transgenesis. However, insofar as parameters i–iv have been used individually in the creation of APP Tg mice (8, 17–19, 22, 23), it is plausible that a synergism between all four parameters contributes to the desirable properties of TgCRND8 mice.

Biochemistry of TgCRND8 Mice—With the possible exception of the Tg19959 line, TgCRND8 mice are currently unique

TABLE II
Amyloid deposition in APP transgenic mice

Tg line	Aβ42 pmol/g[a]	Aβ40	Aβ42/40	Plaque onset	Ref.
	age months[b]	*pmol/g*[c]		*months*	
PDAPP	615 ± 333 (12)	~79[c]	~7.8	6–9	17
Tg2576	175 ± 26 (11–13)	264 ± 38	0.7	9	18
TgAPP/V717I[d]	~170 (15)	~440	~0.4	13	73
TgCRND8	4,600 ± 1,560 (6)	2,440 ± 350	2.0	3	This paper, (1)
	3200 ± 350[e] (6)	920 ± 125	3.5		

[a] Results of Aβ ELISAs are expressed per g (wet weight of brain). Transgenic mice are listed chronologically. Some values derive from formic acid extraction, whereas others use a guanidinium-hydrochloride procedure. Some values were converted from ng to g using values of 4328 and 4513 Da for Aβ 40 and 42, respectively.

[b] Except for the TgAPP/V717I line, all Aβ values are reported for a time point 3 months after the first onset of plaque deposition, as determined histologically. These time points are given in months (in parentheses).

[c] Aβ40 was calculated by subtraction of Aβ42 from total Aβ values.

[d] ELISA values represent plaque-associated (insoluble) Aβ.

[e] Second set of ELISA values derive from a control group of TgCRND8 mice immunized with islet-associated polypeptide (1).

among single transgenic APP mice with regard to the severity of Aβ deposition. Histological deposition of Aβ in amyloid plaques in TgCRND8 mice is evident in 100% of animals by 3 months after birth (n = 28), earlier than in APP transgenic lines described previously (17–19, 49). These results find a parallel in measurements of Aβ40 and Aβ42 derived from ELISA assays (Tables I and II); for example, levels of Aβ42 in 6-month-old TgCRND8 mice approximate those seen in PDAPP mice at 16 months of age (31). Furthermore, two results emerging from these ELISAs show a direct parallel to AD pathogenesis and bear particular emphasis. First, the levels of total Aβ in TgCRND8 mice at 6 months of age, 3,200–4,600 pmol/g brain (as determined by two independent ELISA configurations employing different antibodies), fall into the range observed for sporadic AD cases, 500–5000 pmol/g wet tissue (50). Second, the ratio of Aβ42 to Aβ40 exceeds unity, as is also the case in sporadic AD (50) (and in PDAPP mice expressing a V717F mutation (31)). Insofar as Aβ42 is thought to be the most aggregation-prone and toxic form of Aβ, the skew toward the production of Aβ42 apparent in these mice presumably contributes to the unusually early onset of amyloid deposition.

Although the combined effect of the two FAD mutations affecting both β- and γ-secretase processing of APP is presumed to be a powerful determinant of this potent amyloidogenesis, it is notable that other "double mutant" TgAPP mice only show 100% of animals positive for plaques at 8–10, 18, or 21–25 months of age (19, 49, 51). Although the degree of APP overexpression could also prove crucial in distinguishing TgCRND8 mice from other double mutant TgAPP lines, further variables include PrP, *thy-1,* or platelet-derived growth factor-β promoters with different tropisms and different APP coding region cassettes (APP695, APP751, or intron-containing cassettes capable of producing all three APP isoforms).

Finally, it is remarkable that even though TgCRND8 mice exhibit a high basal synthesis of Aβ, levels of this peptide can be elevated to yet higher levels by mutant versions of PS1. Importantly, two PS1 mutations in *cis* shown to act in an additive way in transfected cells (46) behave in a similar fashion *in vivo*. Thus, double transgenic mice incorporating 4 FAD mutations can develop Aβ deposits by 1 month of age (Fig. 5*B*).

Neuropathology in Transgenic Models of Alzheimer's Disease—TgCRND8 mice exhibit AD-like amyloid plaque deposits with a variety of morphologies. Dense-cored deposits are present from an early stage, and the majority of these (>80%) can be stained with either Congo Red or thioflavine S. With aging, the plaques become larger and multicentric dense-cored deposits appear. Diffuse Aβ immunostaining is also apparent at later stages, being particularly prominent after formic acid pretreatment. Diffuse staining is evident as a penumbra around large plaques and also in the form of isolated deposits (*i.e.* not obviously associated with plaques) throughout the neuropil. From 5 months of age the mature plaques in TgCRND8 mice exhibit neuritic changes strikingly similar to those seen in AD (Fig. 4). Dystrophic neurites are revealed by silver impregnation or NF-200 immunostaining, with dystrophic boutons visualized by synaptophysin or ubiquitin antibodies. Astrocytes and microglial cells often encircle dense-cored deposits, indicating the plaques are capable of initiating an inflammatory response. The other pathological hallmarks of AD are generally accepted to include neuronal loss and the accumulation of NFTs. Focal neuronal loss has been reported in only one Tg model of AD (20, 52, 53), and analogous studies in TgCRND8 mice are underway. NFTs are absent in TgCRND8 mice, as indeed they are in other TgAPP mice. It is possible coding sequence divergence between mouse and human may be of importance, and expression of human tau and cognate tau kinases, perhaps the p25 fragment of p35 (54, 55) or glycogen synthese kinase 3β, may be required to fully recapitulate this pathology.

The Origins of Cognitive Dysfunction in Alzheimer's Disease—In Alzheimer's disease patients, pathological changes detected post-mortem are foreshadowed in the clinical presentation by erosion of mental function leading to frank dementia. Therefore, plausible animal models of AD should develop cognitive deficits at least by the time of appearance of AD-related neuropathology. TgCRND8 mice fulfill this expectation. They represent an example of mice expressing full-length APP where deficits in acquisition of spatial reference memory are present at the onset of AD-related neuropathology (Fig. 3) (1) and thus exhibit some similarities to Tg2576 mice (18, 24). In studies of other TgAPP mice, impaired performance in the water maze test preceded neuropathological changes (23) or was not reported (19). Impaired performance in other testing paradigms has been reported in PDAPP V717F mice, but here a subset of cognitive deficits was better correlated to confounding neuroanatomical abnormalities than to AD-related pathologies (21, 25). In the most recent studies, an age-related deficit in learning capacity was detected in PDAPP mice using a new water maze testing regimen (56). Nonetheless, from a practical point of view, the cognitive deficits in TgCRND8 mice have three important attributes as follows: they occur early in life; they are easily detected in the conventional spatial reference memory version of the water maze; and they are sufficiently robust to be detected without recourse to large sample sizes.

Although overaccumulation of the Aβ peptide is firmly implicated in AD pathogenesis, the mechanisms leading to cognitive decline are not clear. As attempts to correlate plaque burdens and cognitive deterioration have produced mixed results (see Refs. 57–65 and also see Ref. 56), it is possible Aβ affects the central nervous system by mechanisms other than (or in addition to) the toxicity of extracellular, aggregated forms. Indeed, recent studies emphasize soluble forms of Aβ as crucial determinants of clinical outcome (50, 66, 67). Unfortunately, the molecular determinants for neurotoxicity (for example, free radical generating capacity of metal-bound Aβ42 (68, 69) and perturbed signal transduction (70)) and the cellular consequences thereof (altered synaptic function, excitotoxicity, and induction of apoptosis (49, 66)) are undetermined. We suggest TgCRND8 mice comprise a useful and validated system to address these issues. Amelioration of cognitive deficits by immunization against Aβ42 peptide (1) provides compelling evidence for a strong pathogenic role for Aβ peptide in the TgCRND8 model of AD and indeed for the amyloid cascade hypothesis. Parenthetically, these data effectively exclude the proposition that cognitive deficits in TgCRND8 mice derive from an insertional mutation. Furthermore, synthesis of antisera in Aβ42-immunized mice that react strongly with the peptide presented in a β-sheet conformation (1) suggests a potential to dissect the mechanism of Aβ neuropathogenesis (for example, by passive immunization (71) with conformation-specific antibodies). In short, the availability of a new AD model with robust cognitive deficits, levels of Aβ peptide that equal those seen in AD cases, and AD-like pathology may allow both an improved understanding of causal relationships between these phenotypic traits and testing of candidate interventions.

Acknowledgments—We thank Richard Rubenstein for generously supplying the 4G8 antibody, Frédéric Checler for the 3542 antibody, Sam Gandy for the 369 antibody, Julie Panakos for DNA microinjections, and Isabelle Aubert for discussions.

REFERENCES

1. Janus, C., Pearson, J., McLaurin, J., Mathews, P. M., Jiang, Y., Schmidt, S. D., Chishti, M. A., Horne, P., Heslin, D., French, J., Mount, H. T. J., Nixon, R. A., Mercken, M., Bergereon, C., Fraser, P. E., St. George-Hyslop, P., and

Westaway, D. (2000) *Nature* **408,** 979–982
2. Citron, M., Oltersdorf, T., Haass, C., McConlogue, C., Hung, A. Y., Seubert, P., Vigo-Pelfrey, C., Lieberburg, I., and Selkoe, D. J. (1992) *Nature* **360,** 672–674
3. Roher, A. E., Lowenson, J. D., Clarke, S., Wolkow, C., Wang, R., Cotter, R. J., Reardon, I. L., Zurcher-Neely, H. A., Heinrikson, R. L., Ball, M. J., and Greenberg, B. D. (1993) *J. Biol. Chem.* **268,** 3072–3083
4. Johnston, J. A., Cowburn, R. F., Norgren, S., Wiehager, B., Venizelos, N., Winblad, B., Vigo-Pelfrey, C., Schenk, D., Lannfelt, L., and O'Neill, C. (1994) *FEBS Lett.* **354,** 274–278
5. Suzuki, N., Cheung, T. T., Cai, X.-D., Odaka, A., Otvos, L., Eckman, C., Golde, T., and Younkin, S. G. (1994) *Science* **264,** 1336–1340
6. Thinakaran, G., Teplow, D. B., Siman, R., Greenberg, B., and Sisodia, S. S. (1996) *J. Biol. Chem.* **271,** 9390–9397
7. Duff, K., Eckman, C., Zehr, C., Yu, X., Prada, C.-M., Perez-tur, J., Hutton, M., Buee, L., Harigaya, Y., Yager, D., Morgan, D., Gordon, M., Holcomb, L., Refolo, L., Zenk, B., Hardy, J., and Younkin, S. (1996) *Nature* **383,** 710–713
8. Borchelt, D. R., Thinakaran, G., Eckman, C. B., Lee, M. K., Davenport, F., Ratovitsky, T., Prada, C.-M., Kim, G., Seekins, S., Yager, D., Slunt, H. H., Wang, R., Seeger, M., Levey, A. I., Gandy, S. E., Copeland, N. G., Jenkins, N. A., Price, D. L., Younkin, S. G., and Sisodia, S. S. (1996) *Neuron* **17,** 1005–1013
9. Citron, M., Westaway, D., Xia, W., Carlson, G. A., Diehl, T., Levesque, G., Johnson-Wood, K., Lee, M., Seubert, P., Davis, A., Kholodenko, D., Motter, R., Sherrington, R., Perry, B., Yao, H., Strome, R., Lieberburg, I., Rommens, J., Kim, S., Schenk, D., Fraser, P., St. George-Hyslop, and Selkoe, D. (1997) *Nat. Med.* **3,** 67–72
10. Corder, E. H., Saunders, A. M., Srittmatter, W. J., Schmechel, D. E., Gaskell, P. C., Small, G. W., Roses, A. D., Haines, J. L., and Pericak-Vance, M. A. (1993) *Science* **261,** 921–923
11. Holtzman, D. M., Bales, K. R., Tenkova, T., Fagan, A. M., Parsadanian, M., Sartorius, L. J., Mackey, B., Olney, J., McKeel, D., Wozniak, D., and Paul, S. M. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 2892–2897
12. Yankner, B. A., Duffy, L. K., and Kirschner, D. A. (1990) *Science* **250,** 279–282
13. Lorenzo, A., and Yankner, B. A. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 12243–12247
14. Geula, C., Wu, C. K., Saroff, D., Lorenzo, A., Yuan, M., and Yankner, B. A. (1998) *Nat. Med.* **4,** 827–831
15. Hsiao, K. (1998) *Exp. Gerontol.* **33,** 883–889
16. Janus, C., Chishti, M. A., and Westaway, D. (2000) *Biochim. Biophys. Acta* **1502,** 63–75
17. Games, D., *et al.* (1995) *Nature* **373,** 523–527
18. Hsiao, K. H., Chapman, P., Nilsen, S., Eckman, C., Harigawa, Y., Younkin, S., Yang, F. S., and Cole, G. (1996) *Science* **274,** 99–102
19. Sturchler-Pierrat, C., Abramowski, D., Duke, M., Wiederhold, K. H., Mistl, C., Rothacher, S., Ledermann, B., Burki, K., Frey, P., Paganetti, P. A., Waridel, C., Calhoun, M. E., Jucker, M., Probst, A., Staufenbiel, M., and Sommer, B. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 13287–13292
20. Calhoun, M. E., Wederhold, K.-H., Abramowski, D., Phinney, A. L., Probst, A., Sturchler-Pierrat, C., Staufenbiel, M., Sommer, B., and Jucker, M. (1998) *Nature* **395,** 755–756
21. Dodart, J. C., Mathis, C., Saura, J., Bales, K. R., Paul, S. M., and Ungerer, A. (2000) *Neurobiol. Dis.* **7,** 71–85
22. Hsiao, K. K., Borchelt, D. R., Olson, K., Johannsdottir, R., Kitt, C., Yunis, W., Xu, S., Eckman, C., Younkin, S., Price, D., Iadecola, C., Clark, H. B., and Carlson, G. A. (1995) *Neuron* **15,** 1203–1218
23. Moechars, D., Dewachter, I., Lorent, K., Reverse, D., Baekelandt, V., Naidu, A., Tesseur, I., Spittaels, K., Haute, C. V., Checler, F., Godaux, E., Cordell, B., and Van Leuven, F. (1999) *J. Biol. Chem.* **274,** 6483–6492
24. Chapman, P. F., White, G. L., Jones, M. W., Cooper-Blacketer, D., Marshall, V. J., Irizarry, M., Younkin, L., Good, M. A., Bliss, T. V., Hyman, B. T., Younkin, S. G., and Hsiao, K. K. (1999) *Nat. Neurosci.* **2,** 271–276
25. Dodart, J. C., Meziane, H., Mathis, C., Bales, K. R., Paul, S. M., and Ungerer, A. (1999) *Behav. Neurosci.* **113,** 982–990
26. Kang, J., Lemaire, H.-G., Unterbeck, A., Salbaum, J. M., Masters, C. L., Grzeschik, K.-H., Multhaup, G., Beyreuther, K., and Muller-Hill, B. (1987) *Nature* **325,** 733–736
27. Vieira, J., and Messing, J. (1982) *Gene (Amst.)* **19,** 259–268
28. Bolivar, F., Rodriguez, R. L., Greene, P. J., Betlach, M. C., Heyneker, H. L., and Boyer, H. W. (1977) *Gene (Amst.)* **2,** 95–113
29. Scott, M. R., Köhler, R., Foster, D., and Prusiner, S. B. (1992) *Protein Sci.* **1,** 986–997
30. Scott, M., Foster, D., Mirenda, C., Serban, D., Coufal, F., Wälchli, M., Torchia, M., Groth, D., Carlson, G., DeArmond, S. J., Westaway, D., and Prusiner, S. B. (1989) *Cell* **59,** 847–857
31. Johnson-Wood, K., Lee, M., Motter, R., Hu, K., Gordon, G., Barbour, R., Khan, K., Gordon, M., Tan, H., Games, D., Lieberburg, I., Schenk, D., Seubert, P., and McConlogue, L. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 1550–1555
32. Haccou, P., and Meelis, E. (1995) *Statistical Analysis of Behavioural Data*, pp. 120–186 Oxford University Press, Oxford
33. Janus, C., D'Amelio, S., Amitay, O., Chishti, M. A., Strome, R., Fraser, P. E., Carlson, G. A., Roder, J., St. George-Hyslop, P., and Westaway, D. (2000) *Neurobiol. Dis.* **21,** 541–549
34. Gass, P., Wolfer, D. P., Balschun, D., Rudolph, D., Frey, U., Lipp, H. P., and Schutz, G. (1998) *Learn. Mem.* **5,** 274–88
35. Wehner, J. M., Sleight, S., and Upchurch, M. (1990) *Brain Res.* **523,** 181–187
36. Carlson, G. A., Borchelt, D. R., Dake, A., Turner, S., Danielson, V., Coffin, J. D., Eckman, C., Meiners, J., Nilsen, S. P., Younkin, S. G., and Hsiao, K. K. (1997) *Hum. Mol. Genet.* **6,** 1951–1959
37. Iadecola, C., Zhang, F., Niwa, K., Eckman, C., Turner, S. K., Fischer, E., Younkin, S., Borchelt, D. R., Hsiao, K. K., and Carlson, G. A. (1999) *Nat. Neurosci.* **2,** 157–161
38. Telling, G. C., Scott, M., Mastrianni, J., Gabizon, R., Torchia, M., Cohen, F. E., DeArmond, S. J., and Prusiner, S. B. (1995) *Cell* **83,** 79–90
39. Moser, M., Colello, R. J., Pott, U., and Oesch, B. (1995) *Neuron* **14,** 509–517
40. DeArmond, S. J., Sanchez, H., Yehiely, F., Qiu, Y., Ninchak-Casey, A., Daggett, V., Camerino, A. P., Cayetano, J., Rogers, M., Groth, D., Torchia, M., Tremblay, P., Scott, M. R., Cohen, F. E., and Prusiner, S. B. (1997) *Neuron* **19,** 1337–1348
41. Irizarry, M. C., McNamara, M., Fedorchak, K., Hsiao, K., and Hyman, B. T. (1997) *J. Neuropathol. Exp. Neurol.* **56,** 965–973
42. Kim, K. W., Miller, D. L., Sapienza, V. J., Chen, C. M. J., Bai, C., Grundke-Iqbal, I., Currie, J. R., and Wisnieski, H. M. (1988) *Neurosci. Res. Commun* **2,** 121–130
43. Spillantini, M. G., Goedert, M., Jakes, R., and Klug, A. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 3947–3951
44. Oesch, B., Westaway, D., Wälchli, M., McKinley, M. P., Kent, S. B. H., Aebersold, R., Barry, R. A., Tempst, P., Teplow, D. B., Hood, L. E., Prusiner, S. B., and Weissmann, C. (1985) *Cell* **40,** 735–746
45. Braak, H., and Braak, E. (1991) *Acta Neuropathol.* **82,** 239–259
46. Citron, M., Eckman, C. B., Diehl, T. S., Corcoran, C., Ostaszewski, B. L., Xia, W., Levesque, G., St. George-Hyslop, P., Younkin, S. G., and Selkoe, D. J. (1998) *Neurobiol. Dis.* **5,** 107–116
47. Storey, E., and Cappai, R. (1999) *Neuropathol. Appl. Neurobiol.* **25,** 81–97
48. Sherrington, R., Rogaev, E., Liang, Y., Rogaeva, E., Levesque, G., Ikeda, M., Chi, H., Lin, C., Holman, K., Tsuda, T., Mar, L., Fraser, P., Rommens, J. M., and St. George-Hyslop, P. (1995) *Nature* **375,** 754–760
49. Hsia, A. Y., Masliah, E., McConlogue, L., Yu, G. Q., Tatsuno, G., Hu, K., Kholodenko, D., Malenka, R. C., Nicoll, R. A., and Mucke, L. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 3228–3233
50. Wang, J., Dickson, D. W., Trojanowski, J. Q., and Lee, V. M. (1999) *Exp. Neurol.* **158,** 328–337
51. Mucke, L., Masliah, E., Yu, G. Q., Mallory, M., Rockenstein, E. M., Tatsuno, G., Hu, K., Kholodenko, D., Johnson-Wood, K., and McConlogue, L. (2000) *J. Neurosci.* **20,** 4050–4058
52. Masliah, E., Sisk, A., Mallory, M., Mucke, L., Schenk, D., and Games, D. (1996) *J. Neurosci.* **16,** 5795–5811
53. Irizarry, M. C., McNamara, M., Page, K. J., Schenk, D., Games, D., and Hyman, B. T. (1997) *J. Neurosci.* **17,** 7053–7059
54. Patrick, G. N., Zukerberg, L., Nikolic, M., de la Monte, S., Dikkes, P., and Tsai, L. H. (1999) *Nature* **402,** 615–622
55. Ahlijanian, M. K., Barrezueta, N. X., Williams, R. D., Jakowski, A., Kowsz, K. P., McCarthy, S., Coskran, T., Carlo, A., Seymour, P. A., Burkhardt, J. E., Nelson, R. B., and McNeish, J. D. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 2910–2915
56. Chen, G., Chen, K. S., Knox, J., Inglis, J., Bernard, A., Martin, S. J., Justice, A., McConlogue, L., Games, D., Freedman, S. B., and Morris, R. G. (2000) *Nature* **408,** 975–979
57. Masliah, E., Terry, R. D., Alford, M., DeTeresa, R. M., and Hansen, L. A. (1991) *Am. J. Pathol.* **138,** 235–246
58. Terry, R. D., Masliah, E., Salmon, D. P., Butters, N., DeTeresa, R., Hill, R., Hansen, L. A., and Katzman, R. (1991) *Ann. Neurol.* **30,** 572–580
59. Arriagada, P. V., Growdon, J. H., Hedley-Whyte, E. T., and Hyman, B. T. (1992) *Neurology* **42,** 631–639
60. Terry, R. D. (1996) *J. Neuropathol. Exp. Neurol.* **55,** 1023–1025
61. Cummings, B. J., Pike, C. J., Shankle, R., and Cotman, C. W. (1996) *Neurobiol. Aging* **17,** 921–933
62. Gomez-Isla, T., Hollister, R., West, H., Mui, S., Growdon, J. H., Petersen, R. C., Parisi, J. E., and Hyman, B. T. (1997) *Ann. Neurol.* **41,** 17–24
63. Bartoo, G. T., Nochlin, D., Chang, D., Kim, Y., and Sumi, S. M. (1997) *J. Neuropathol. Exp. Neurol.* **56,** 531–540
64. Crook, R., Verkkoneimi, A., Perez-Tur, J., Mehta, N., Baker, M., Houlden, H., Farrer, M., Hutton, M., Lincoln, S., Hardy, J., Gwinn, K., Somer, M., Paetau, A., Kalimo, H., Ylikoski, R., Poyhonen, M., Kucera, S., and Haltia, M. (1998) *Nat. Med.* **4,** 452–455
65. Beach, T. G., Kuo, Y. M., Spiegel, K., Emmerling, M. R., Sue, L. I., Kokjohn, K., and Roher, A. E. (2000) *J. Neuropathol. Exp. Neurol.* **59,** 308–313
66. Lue, L. F., Kuo, Y. M., Roher, A. E., Brachova, L., Shen, Y., Sue, L., Beach, T., Kurth, J. H., Rydel, R. E., and Rogers, J. (1999) *Am. J. Pathol.* **155,** 853–862
67. McLean, C. A., Cherny, R. A., Fraser, F. W., Fuller, S. J., Smith, M. J., Beyreuther, K., Bush, A. I., and Masters, C. L. (1999) *Ann. Neurol.* **46,** 860–866
68. Huang, X., Atwood, C. S., Hartshorn, M. A., Multhaup, G., Goldstein, L. E., Scarpa, R. C., Cuajungco, M. P., Gray, D. N., Lim, J., Moir, R. D., Tanzi, R. E., and Bush, A. I. (1999) *Biochemistry* **38,** 7609–7616
69. Huang, X., Cuajungco, M. P., Atwood, C. S., Hartshorn, M. A., Tyndall, J. D., Hanson, G. R., Stokes, K. C., Leopold, M., Multhaup, G., Goldstein, L. E., Scarpa, R. C., Saunders, A. J., Lim, J., Moir, R. D., Glabe, C., Bowden, E. F., Masters, C. L., Fairlie, D. P., Tanzi, R. E., and Bush, A. I. (1999) *J. Biol. Chem.* **274,** 37111–37116
70. Harkany, T., Abraham, I., Konya, C., Nyakas, C., Zarandi, M., Penke, B., and Luiten, P. G. (2000) *Rev. Neurosci.* **11,** 329–382
71. Bard, F., Cannon, C., Barbour, R., Burke, R.-L., Games, D., Grajeda, H., Guido, T., Hu, K., Huang, J., Johnson-Wood, K., Khan, K., Kholodenko, D., Lee, M., Lieberburg, I., Motter, R., Nguyen, M., Soriano, F., Vasquez, N., Weiss, K., Welch, B., Seubert, P., Schenk, D., and Yednock, T. (2000) *Nat. Med.* **6,** 916–919
72. Capell, A., Saffrich, R., Olivo, J.-C., Meyn, L., Walter, J., Grunberg, J., Dotti, C., and Haass, C. (1997) *J. Neurochem.* **69,** 2432–2340
73. Dewachter, I., Van Dorpe, J., Smeijers, L., Gilis, M., Kuiperi, C., Laenen, I., Caluwaerts, N., Moechars, D., Checler, F., Vanderstichele, H., and Van Leuven, F. (2000) *J. Neurosci.* **20,** 6452–6458

nature 21/28th December 2000

letters to nature

Nature **408**, 979 - 982 (2000); doi:10.1038/35050110

Aβ peptide immunization reduces behavioural impairment and plaques in a model of Alzheimer's disease

CHRISTOPHER JANUS*, JACQUELINE PEARSON*, JOANNE MCLAURIN*, PAUL M. MATHEWS†, YING JIANG†, STEPHEN D. SCHMIDT†, M. AZHAR CHISHTI*, PATRICK HORNE*, DONNA HESLIN*, JANET FRENCH*, HOWARD T.J. MOUNT*, RALPH A. NIXON†, MARC MERCKEN‡, CATHERINE BERGERON*§, PAUL E. FRASER*, PETER ST GEORGE-HYSLOP*§ & DAVID WESTAWAY*

* Centre for Research in Neurodegenerative Diseases, Departments of Medicine, Laboratory Medicine and Pathobiology, and Medical Biophysics, University of Toronto, Tanz Neuroscience Building, 6 Queen's Park Crescent West, Toronto, Ontario M5S 3H2, Canada
† Nathan Kline Institute Center for Dementia Research, and New York University School of Medicine, 140 Old Orangeburg Road, Orangeburg, New York 10962, USA
‡ Janssen Research Foundation, Turnhoutseweg, 30, B-2340 Beerse, Belgium
§ Departments of Medicine (Division of Neurology) and Pathology, Toronto Western Hospital, University Health Network , Toronto, Ontario M5S 1A8, Canada

Correspondence and requests for materials should be addressed to P.H.StG.-H. (e-mail: p.hyslop@utoronto.ca).

Much evidence indicates that abnormal processing and extracellular deposition of amyloid-β peptide (Aβ), a proteolytic derivative of the β-amyloid precursor protein (βAPP), is central to the pathogenesis of Alzheimer's disease (reviewed in ref. 1). In the PDAPP transgenic mouse model of Alzheimer's disease, immunization with Aβ causes a marked reduction in burden of the brain amyloid. Evidence that Aβ immunization also reduces cognitive dysfunction in murine models of Alzheimer's disease would support the hypothesis that abnormal Aβ processing is essential to the pathogenesis of Alzheimer's disease, and would encourage the development of other strategies directed at the 'amyloid cascade'. Here we show that Aβ immunization reduces both deposition of cerebral fibrillar Aβ and cognitive dysfunction in the TgCRND8 murine model of Alzheimer's disease without, however, altering total levels of Aβ in the brain. This implies that either a ~50% reduction in dense-cored Aβ plaques is sufficient to affect cognition, or that vaccination may modulate the activity/abundance of a small subpopulation of especially toxic Aβ species.

Supplementary Information for more details) in serum samples (200 μl of blood) collected at 13 and 25 weeks.

Behavioural tests and data analysis

The water maze apparatus, mouse handling and general testing procedures have been described[23]. Before the first spatial learning test at 11 weeks, all mice underwent non-spatial pre-training (NSP) to assess swimming abilities and to accustom mice to the test[24,25] (see Supplementary Information). Two days after the NSP phase, all mice underwent a reference memory training with a hidden platform placed in the centre of one quadrant of the pool for 5 days, with four trials per day. After the last trial of day 5, the platform was removed from the pool and each mouse received one 60-s swim probe trial. Escape latency (s), length of swim path (cm), swim speed (cm s^{-1}), % of floating (speed less then 5 cm s^{-1}), % of time in outer zone (near the pool wall), and % of time and path in each quadrant of the pool were recorded using an on-line HVS image video tracking system[23] (see Supplementary Information).

For the probe trials, an annulus-crossing index was calculated that represents the number of passes over the platform site, minus the mean of passes over alternative sites in other quadrants. The index expresses the spatial place preference and controls for alternative search strategies without place preferences, such as circular search paths[26,27]. All mice were re-tested at 15, 19 and 23 weeks of age, one week before the next immunization. At each re-testing, the platform was placed in the centre of a different, semi-randomly chosen pool quadrant for all five sessions of training. At the end of the experiment, all mice were given a cue (visual platform) learning test. This was followed by the open-field test to investigate spontaneous locomotor exploration. Behavioural data was analysed using a mixed model of factorial ANOVA. Degrees of freedom were adjusted by Greenhouse–Geisser epsilon correction for heterogeneity of variance. A Bonferroni Inequality correction was applied for multiple comparisons. Omega squared (ω^2) was used as a measure of effect size caused by different factors.

Analysis of βAPP and amyloid burden in brain

Three 5-μm sections at 25-μm intervals from one cerebral hemisphere were immunostained with Dako 6F/3D anti-Aβ monoclonal antibody to residues 8–17 (which is primarily reactive against dense-cored plaques) with 4G8 (ref. 28), or with sera from immunized mice, and counterstained with haematoxylin and resin mounted as described (M.A.C. *et al.*, manuscript in preparation). For some samples the formic-acid treatment step was omitted. End products were visualized with diaminobenzidine. Amyloid plaque burden was assessed using Leco IA-3001 image analysis software interfaced with a Leica microscope and a Hitachi KP-M1U CCD video camera. The quantitative analysis was performed at of × 25 magnification, and the image frame and guard size was set to 0,0,639,479 (307,200 μm^2) for each slide. The brain area (cortex or hippocampus) was outlined using the edit plane function, and the area and number of plaques in the outlined structure were recorded. Data were pooled for all three sections.

Cerebral Aβ levels were assayed from formic-acid-extracted[29], hemi-brain sucrose homogenates using an ELISA method (see Supplementary Information) in which Aβ was trapped with either monoclonal antibody to $A\beta_{40}$ (JRF/cAb40/10) or $A\beta_{42}$ (JRF/cAb42/26) and then detected with horseradish peroxidase (HRP)-conjugated JRF/Abtot/17. The dilution of JRF/Abtot/17 and samples were optimized to detect Aβ in the range of 50 to 800 fmol ml^{-1}. ELISA signals are reported as the mean ± s.e.m. of four replica wells in fmol Aβ per mg total protein (determined with the BioRad DC protein assay), based on standard curves using synthetic $A\beta_{1-40}$ and $A\beta_{1-42}$ peptide standards (American Peptide Co. Sunnyvale, CA). Cerebral βAPPs levels were analysed in supernatant of brain as described[30].

Received 19 July; accepted 16 November 2000.

1. Steiner, H., Capell, A., Leimer, U. & Haass, C. Genes and mechanisms involved in beta-amyloid generation and Alzheimer's disease. *Eur. Arch. Psychiat. Clin. Neurosci.* **249**, 266–270 (1999).
2. Schenk, D. *et al.* Immunization with Aβ attenuates Alzheimer's disease-like pathology in the PDAPP mouse. *Nature* **400**, 173–177 (1999).
3. Bard, F. *et al.* Peripherally administered antibodies against amyloid beta-peptide enter the central nervous system and reduce pathology in a mouse model of Alzheimer disease. *Nature Med.* **6**, 916–919 (2000).
4. Whitehouse, P. J. *et al.* Clinical trial designs for demonstrating disease-course-altering effects in dementia. *Alzheimer Dis. Assoc. Disord.* **12**, 281–294 (1998).
5. Morris, R. Developments of a water-maze procedure for studying spatial learning in the rat. *J. Neurosci. Methods* **11**, 47–60 (1984).
6. Markowska, A. L., Long, J. M., Johnson, C. T. & Olton, D. S. Variable-interval probe test as a tool for repeated measurements of spatial memory in the water maze. *Behav. Neurosci.* **107**, 627–632 (1993).
7. Martin, P. & Bateson, P. *Measuring Behaviour* (Cambridge Univ. Press, Cambridge, 1996).
8. Hsiao, K. Correlative memory deficits, Aβ elevation, and amyloid plaques in transgenic mice. *Science* **274**, 99–102 (1996).
9. Rogers, S. L., Farlow, M. R., Doody, R. S., Mohs, R. & Friedhoff, L. T. A 24-week, double-blind, placebo-controlled trial of donepezil in patients with Alzheimer's disease. Donepezil study group. *Neurology* **50**, 136–145 (1998).
10. Terry, R. D., Masliah, E. & Hansen, L. A. in *Alzheimer Disease* 2nd edn (eds Terry, R. D., Katzman, R., Bick, K. C. & Sisodia, S. S.) (Lippincott Williams Wilkins, Philadelphia, 1999).
11. Moechars, D. *et al.* Early phenotypic changes in transgenic mice that over-express different mutants of amyloid precursor protein. *J. Biol. Chem.* **274**, 6483–6492 (1999).
12. Hsia, A. Y. Plaque-independent disruption of neural circuits in Alzheimer's disease mouse models. *Proc. Natl Acad. Sci. USA* **96**, 3228–3233 (1999).
13. Solomon, B., Koppel, R., Hanan, E. & Katzav, T. Monoclonal antibodies inhibit in vitro fibrillar aggregation of the Alzheimer β-amyloid peptide. *Proc. Natl Acad. Sci. USA* **93**, 452–455 (1996).
14. Lorenzo, A. & Yanker, B. A. β-amyloid neurotoxicity requires fibril formation and is inhibited by Congo red. *Proc. Natl Acad. Sci. USA* **91**, 12243–12247 (1994).
15. Walsh, D. M. *et al.* Amyloid β-protein fibrillogenesis. Structure and biological activity of protofibrillar intermediates. *J. Biol. Chem.* **274**, 25945–25952 (1999).
16. Hartley, D. M. Protofibrillar intermediates of amyloid β-protein induce acute electrophysiological changes and progressive neurotoxicity in cortical neurons. *J. Neurosci.* **19**, 8876–8884 (1999).
17. McLean, C. A. *et al.* Soluble pool of Aβ amyloid as a determinant of severity of neurodegeneration in Alzheimer's disease. *Ann. Neurol.* **46**, 860–866 (1999).
18. Naslund, J. *et al.* Correlation between elevated levels of amyloid β-peptide in the brain and cognitive decline. *J. Am. Med. Assoc.* **283**, 1571–1577 (2000).
19. Mucke, L. *et al.* High-level neuronal expression of Aβ1–42 in wild-type human amyloid protein precursor transgenic mice: synaptotoxicity without plaque formation. *J. Neurosci.* **20**, 4050–4058 (2000).
20. St George-Hyslop, P. & Westaway, D. Alzheimer's disease: antibody clears senile plaques. *Nature* **400**, 116–117 (1999).
21. Lu, D. C. A second cytotoxic proteolytic peptide derived from amyloid β-protein precursor. *Nature Med.* **6**, 397–404 (2000).
22. McLaurin, J., Franklin, T., Chakrabartty, A. & Fraser, P. E. Phosphatidylinositol and inositol involvement in Alzheimer amyloid-beta fibril growth and arrest. *J. Mol. Biol.* **278**, 183–194 (1998).
23. Janus, C. Spatial learning in transgenic mice expressing human presenilin 1 (PS1) transgenes. *Neurobiology of Aging* **21**, 541–549 (2000).
24. Cain, D. P., Beiko, J. & Boon, F. Navigation in the water maze: the role of proximal and visual cues, path intergration, and magnetic field information. *Psychobiology* **25**, 286–293 (1997).
25. Dudchenko, P. A., Goodridge, J. P., Seiterle, D. A. & Taube, J. S. Effects of repeated disorientation on the acquisition of spatial tasks in rats: dissociation between the appetetive radial arm maze and aversive water maze. *J. Exp. Psychol.* **23**, 194–210 (1997).
26. Gass, P. *et al.* Deficits in memory tasks of mice with CREB mutations depend on gene dosage. *Learn Mem.* **5**, 274–288 (1998).
27. Wehner, J. M., Sleight, S. & Upchurch, M. Hippocampal protein kinase C activity is reduced in poor spatial learners. *Brain Res.* **523**, 181–187 (1990).
28. Wisniewski, H. M., Wen, G. Y. & Kim, K. S. Comparison of four staining methods on the detection of neuritic plaques. *Acta Neuropathol.* **78**, 22–27 (1989).
29. Susuki, N. *et al.* An increased percentage of long amyloid B protein secreted by Familial Amyloid β Protein Precursor (BAPP717) mutants. *Science* **264**, 1336–1340 (1994).
30. Citron, M. *et al.* Mutant presenilins of Alzheimer's Disease increase production of 42 residue amyloid β-protein in both transfected cells and transgenic mice. *Nature Med.* **3**, 67–72 (1997).

Supplementary information is available on *Nature*'s World-Wide Web site (http://www.nature.com) or as paper copy from the London editorial office of *Nature*.

Acknowledgements

This work was supported by the Medical Research Council of Canada, Howard Hughes Medical Research Foundation, Alzheimer Society of Ontario, The W. Garfield Weston Foundation and the US National Institute of Aging. We thank G. Carlson for useful discussions, and R. Renlund, K. Parisien, J. Haight and J. Cowieson for help during mice immunization. None of the authors has a financial or other relationship with Elan Pharmaceuticals Inc.

Correspondence and requests for materials should be addressed to P.H.StG.-H. (e-mail: p.hyslop@utoronto.ca).

Aβ peptide vaccination prevents memory loss in an animal model of Alzheimer's disease

Dave Morgan*, David M. Diamond†‡, Paul E. Gottschall*, Kenneth E. Ugen§, Chad Dickey§, John Hardy‖, Karen Duff¶, Paul Jantzen*, Giovanni DiCarlo*, Donna Wilcock*, Karen Connor*, Jaime Hatcher#, Caroline Hope#, Marcia Gordon* & Gary W. Arendash#

** Alzheimer Research Laboratory, Department of Pharmacology, † Department of Psychology, § Department of Medical Microbiology and Immunology, # Alzheimer Research Laboratory, Department of Biology, University of South Florida, Tampa, Florida 33612, USA*

‡ James A. Haley VA Center, Tampa, Florida 33612, USA

‖ Department of Pharmacology, Mayo Clinic, Jacksonville, Florida 32224, USA

¶ Nathan Kline Institute, Orangeburg, New York 10962, USA

Vaccinations with amyloid-β peptide (Aβ) can dramatically reduce amyloid deposition in a transgenic mouse model of Alzheimer's disease[1]. To determine if the vaccinations had deleterious or beneficial functional consequences, we tested eight months of Aβ vaccination in a different transgenic model for

Alzheimer's disease in which mice develop learning deficits as amyloid accumulates[2,3]. Here we show that vaccination with Aβ protects transgenic mice from the learning and age-related memory deficits that normally occur in this mouse model for Alzheimer's disease. During testing for potential deleterious effects of the vaccine, all mice performed superbly on the radial-arm water-maze test of working memory. Later, at an age when untreated transgenic mice show memory deficits, the Aβ-vaccinated transgenic mice showed cognitive performance superior to that of the control transgenic mice and, ultimately, performed as well as nontransgenic mice. The Aβ-vaccinated mice also had a partial reduction in amyloid burden at the end of the study. This therapeutic approach may thus prevent and, possibly, treat Alzheimer's dementia.

The accumulation of fibrils formed from the Aβ peptide into amyloid plaques is a defining characteristic of Alzheimer's disease (AD). The Aβ vaccination protocol described in ref. 1 reduced Aβ deposits, which suggested that this approach might benefit AD patients. However, the functional consequences of such vaccinations might be deleterious. For example, plaque-associated inflammation promoted by the immunization could interfere with normal brain functioning, and/or lead to degenerative changes in the brain[4–8]. We used a novel working-memory task that combines elements of a radial-arm maze and a water maze. This radial-arm water maze is remarkably robust at detecting learning/memory deficits that develop in AD transgenic mice[2] and more efficient in sample size requirements than other memory tasks typically used for rodents[3].

To test the possibility that vaccinations might cause premature memory deficits in AD transgenic mice, we assessed learning/memory performance in the mice at 11.5 months of age after five inoculations with Aβ or the control vaccine, keyhole limpet haemocyanin (KLH). All mice showed strong learning and memory capacity, irrespective of treatment or transgene status (Fig. 1a). All groups averaged three to four errors on the first trial as they sought out the new platform location for that day, but averaged less than one error by trials 4 or 5, demonstrating intact working memory for platform location between trials and during the 30-min delay before trial 5. This strong performance by Aβ-vaccinated mice indicates that any inflammatory responses caused by the vaccine were not deleterious behaviourally.

Monthly inoculations were continued until the mice were 15.5 months, when these mice were tested again in the radial-arm water maze. At 15.5 months the KLH-vaccinated transgenic mice failed to demonstrate learning or memory of the platform location; their performance on all trials was the same (Fig. 1b). This is identical to the performance of other untreated transgenic mice that had been previously tested in this learning task at this age (Fig. 1c; ref. 3). In contrast, the Aβ-inoculated transgenic mice, although slower to learn platform location than nontransgenic mice on trial 3 (Fischer's least

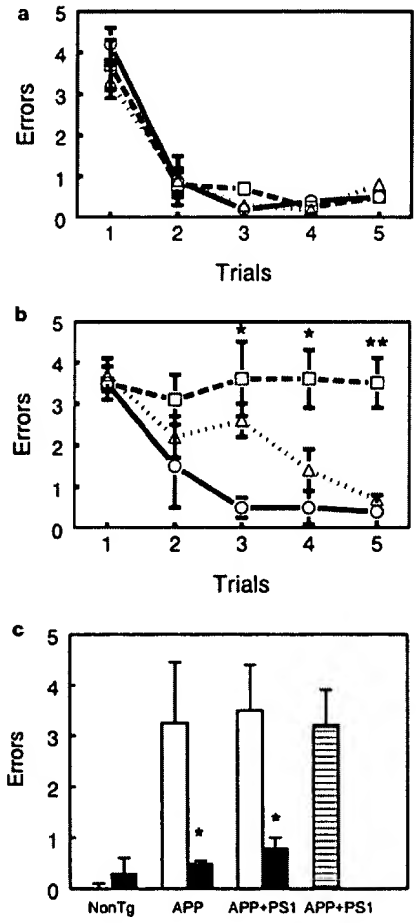

Figure 1 Radial-arm water-maze performance in vaccinated transgenic and nontransgenic mice. **a**, Nontransgenic mice (circles, solid lines), transgenic mice vaccinated with KLH (squares, dashed lines), and transgenic mice vaccinated with Aβ (triangles, dotted lines) were tested in the radial-arm water maze at 11.5 months of age (after five inoculations). All groups learned (trial 4) and remembered (trial 5) the platform location at this time point. In the same mice at 15.5 months of age (nine inoculations; **b**), the transgenic mice vaccinated with Aβ continued to show learning and memory of the platform location, whereas the transgenic mice vaccinated with KLH failed to show learning and memory for platform location on either trials 4 or 5 ($^*P < 0.05$, $^{**}P < 0.01$; KLH significantly different from other two groups by LSD post hoc analysis after MANOVA). This benefit of Aβ vaccination was found in both the APP-only and APP+PS1 transgenic mice (**c**), with significantly fewer errors on trial 5 in the Aβ-vaccinated groups (solid bars) than in the KLH-vaccinated group (open bars) of both genotypes ($^*P < 0.03$). Included for comparison is the trial 5 performance of another group (hatched bars) of untreated 15–16-month-old transgenic mice that were tested separately, and are reported on fully elsewhere[2].

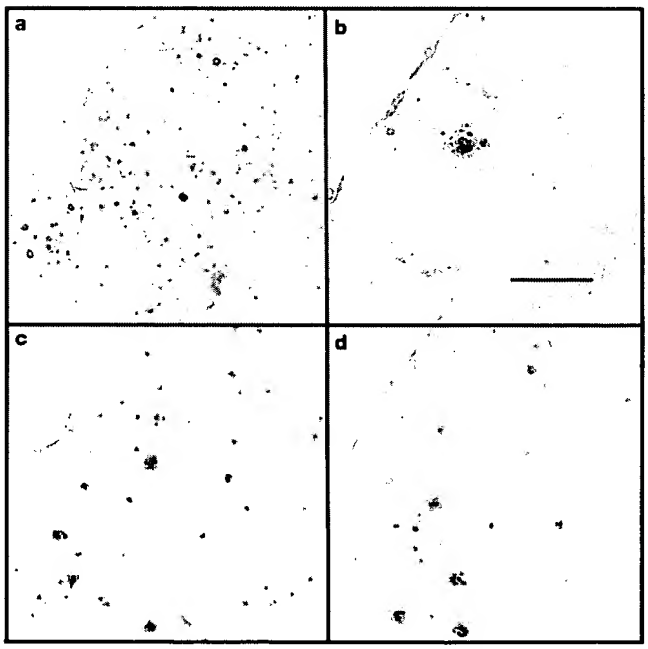

Figure 2 Amyloid pathology in transgenic mice vaccinated with KLH or Aβ. Immunohistochemistry for Aβ in frontal cortex is shown in (KLH-vaccinated) (**a**) and (Aβ-vaccinated) (**b**) in transgenic mice with values similar to the means shown in Fig. 3c. Congo-red staining is shown in (KLH) (**c**) and (Aβ) (**d**) in mice with values corresponding to the means in Fig. 3b. Horizontal sections are oriented with the corpus callosum in the lower right corner and anterior to the top. Scale bar, 500 μm.

significant difference (LSD), $P < 0.02$), were nearly flawless by trial 5, and performed significantly better than the KLH-vaccinated transgenic mice on both trials 4 and 5 (multiple analysis of variance, MANOVA: $F_{(2,15)} = 5.83$, $P < 0.02$ and $F_{(2,15)} = 12.16$, $P < 0.001$, respectively; KLH transgenic group different from both other groups by Fischer's LSD post hoc comparisons, $P < 0.05$ on trial 4 and $P < 0.01$ on trial 5). Our individual evaluation of the performance of the two transgenic genotypes made it clear that both APP-only and APP+PS1 transgenic mice benefited from the Aβ vaccinations (Fig. 1c).

Serological analysis indicated that mice injected with Aβ developed antibodies against the Aβ peptide. Very high titres were found in both transgenic and nontransgenic mice immunized with Aβ (IC_{50} = 27,000 ± 5,000 and 48,000 ± 18,000, respectively; not significant). There was no anti-Aβ activity in the KLH-immunized transgenic mice, untreated transgenic mice, nor nontransgenic mice at final dilutions of serum down to 1:16, indicating that transgenic mice did not spontaneously generate an antibody reaction to Aβ.

Immunization with Aβ caused a modest reduction in Aβ deposits in the frontal cortex, with a significant reduction in the Congo-red-stained area of APP+PS1 mice, and a significant reduction in the Aβ-immunostained area of APP mice (Fig. 2 and Fig. 3). Reductions of a similar extent were found in hippocampus. We also quantified immunostaining using Aβ40- and Aβ42-specific antisera, both of which exhibited the same modest reductions found in total Aβ immunostaining. We suspect that, with a larger sample size, statistically significant partial reductions would be found in all these measures consistent with other recent reports[9–11]. In general, the percentage reduction in Aβ deposition was greater in the APP mice than the APP+PS1 mice. The absolute reductions were greater, however, in the doubly transgenic animals. The APP+PS1 mice already had substantial Aβ deposits by the time vaccinations were initiated[12]. Further studies will test whether beginning vaccinations at an earlier age, or combining vaccination with other Aβ-lowering treatments, will result in more complete protection from Aβ deposition, and improve the cognitive performance of 15-months-old transgenic mice even further.

Our most important finding here is that Aβ vaccination protects transgenic mice from developing memory deficits compared with KLH-immunized (control) transgenic mice. But how important is the learning paradigm in discerning these differences. We have found that in using the reference-memory version of the water maze, mice of this age (15.5 months) have deficits in escape latency, but not retention on the probe trial[3]. Thus, the more demanding working-memory version of the water-maze task may be essential to detect such differences. Similarly, a spatial task would require intact function of hippocampal and, to a lesser extent, cortical structures, the locations where plaques accumulate earliest and to the greatest extent in these mice[12–14].

This vaccination-associated protection from memory impairment occurs in the presence of reduced, but still substantial Aβ deposits. The mechanism by which immunization with Aβ blocks learning and memory deficits is not understood. One possibility is that the antibodies neutralize Aβ in some restricted compartment or deplete a non-deposited form of Aβ (for example, a soluble form) that is responsible for the memory loss observed. Recently, soluble Aβ has been proposed as the cause of synapse loss in APP transgenic mice, as some transgenic lines develop reductions in synaptophysin immunoreactivity in dentate gyrus without developing Aβ deposits[15]. A second possibility is that microglia activated by the inoculations[1] can clear the deposited Aβ, thereby permitting normal cognitive function. This is not easily reconciled with the relatively modest Aβ clearance detected, although exhaustive regional analyses have yet to be completed. Perhaps even mice that have already developed extensive brain pathology and memory deficits can benefit from vaccinations given later in life. In view of the absence of adverse effects on behaviour and brain functioning, and the protection of memory functions by the Aβ vaccines, we strongly recommend testing of this and related approaches for the treatment and prevention of Alzheimer's disease. □

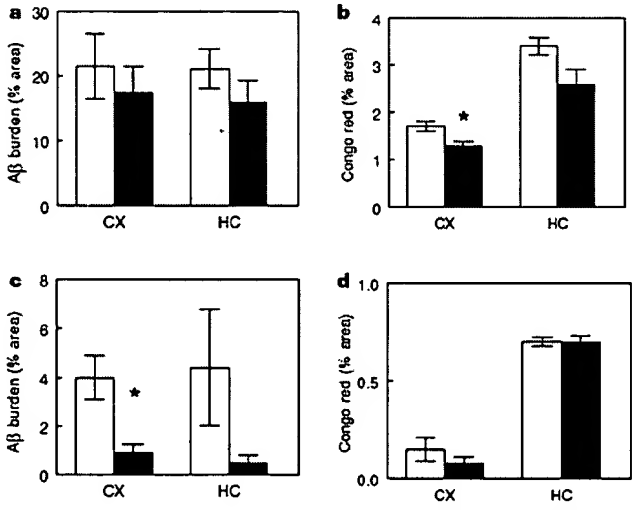

Figure 3 Measurement of amyloid histopathology after Aβ peptide immunization. **a**, **b**, Results for the APP+PS1 mice; **c**, **d**, results for APP-only transgenic mice. A significant reduction in Congo-red staining in frontal cortex was found in APP+PS1 mice vaccinated with Aβ ($n = 4$) compared to in APP+PS1 mice vaccinated with KLH ($n = 5$; **b**). There was a significant reduction in Aβ immunostaining in APP-only transgenic mice vaccinated with Aβ ($n = 3$) compared to in KLH-vaccinated APP mice ($n = 2$; **c**). *, $P < 0.05$; **, $P < 0.01$ by t-test. CX, frontal cortex; HC, hippocampus.

Methods

Vaccination protocols

Mice were obtained by breeding Tg 2576 APP transgenic mice[16] with PS1 line 5.1 transgenic mice[17], resulting in nontransgenic, APP, APP+PS1 and PS1 transgenic mice as described by us previously[12,13]. Human Aβ1–42 peptide (Bachem) was suspended in pyrogen-free Type I water at 2.2 mg ml^{-1} then mixed with 10 × PBS to yield 1 × PBS and incubated overnight at 37 °C. Control mice were injected with KLH that was prepared in the same manner. The antigen suspension was mixed 1:1 with Freund's complete adjuvant and 100 μg Aβ injected subcutaneously by an experimenter who had no role in the behavioural testing. A boost of the same material (prepared freshly) was made in incomplete Freund's at two weeks and injected once monthly for the next three months. Subsequent monthly boosts were made in mineral oil. Mice were vaccinated, beginning at 7.5 months of age. The sample size of each group was: 6 (3 female/3 male) nontransgenic mice vaccinated with Aβ or KLH; 7 (4 female/3 male) transgenic mice vaccinated with Aβ; 7 (4 female/3 male) transgenic mice vaccinated with KLH. The first post-vaccination behavioural testing period was started 5 days after the fifth vaccination at 11.5 months of age. The second behavioural testing period was started at 15.5 months of age, one month after the ninth vaccination. Mice were killed at 16 months of age. We note that transgenic and nontransgenic mice were also tested for performance in the radial-arm water maze at 6 months of age (before vaccination) and all mice performed well.

Radial-arm water maze testing

Experimenters were unaware of the experimental conditions of the mice at the time of testing. The maze consisted of a circular pool 1 m in diameter with six swim alleys (arms) 19 cm wide that radiated out from an open central area (40 cm in diameter), with a submerged escape platform located at the end of one of the arms[3,18]. Spatial cues were present on the walls and ceiling of the testing room. The escape platform was placed in a different arm each day, forcing mice to use working memory to solve the task. Each day, mice were given the opportunity to learn the location of the submerged platform during four consecutive acquisition trials followed 30 min later by a retention trial (trial 5). On each trial, the mouse was started in one arm not containing the platform and allowed to swim for up to one minute to find the escape platform. Upon entering (all four paws within the swim alley) an incorrect arm or failing to select an arm after 20 s, the mouse was gently pulled back to the start arm for that trial and charged an error. All mice spent 30 s on the platform following each trial before beginning the next trial. On subsequent trials that day, the start arm was varied, so the mouse could not simply learn the motor rule 'second arm to the left', but must learn the spatial location of the platform that day. After the fourth trial was completed, the mice were placed in their home cage for 30 min, then returned to the maze and administered the retention trial. The platform was located in the same arm on each trial within a day, and was in a different arm across days. Over 1–2 weeks of

training, control groups gradually improved performance as they learned the procedural aspects of the task, reaching an asymptotic level of 0.5–1 errors on trials 4 and 5. In the experiments presented here mice were trained until the nontransgenic mice reached asymptotic performance: 9 days at 11.5 months or 11 days at 15.5 months. The scores for each mouse on the last two days of testing were averaged and used for statistical analysis. Sensorimotor tests identified no differences among these groups in open field behaviour or string agility testing. As in earlier work, all transgenic mice were impaired on the balance beam, a deficit observed as early as six months of age[3], but this deficit was not modified by Aβ vaccination.

ELISA analysis for serum antibodies

Ninety-six-well Immulon 4HBX (Dynex) micro plates were coated with the Aβ1–42 protein (250 ng per well) for 1 h at 37 °C. They were washed four times with 0.45% NaCl + 0.05% Tween-20 (washing buffer, WB). The plates were blocked with 5% non-fat dry milk (NFDM) in PBS overnight at 4 °C and washed the following day. Mouse serum was prepared in PBS at an initial dilution of 1:16 and subsequent twofold dilutions were made. All samples were run in duplicate and incubated at 37 °C for 1 h followed by washing 10 times in WB. Plates were blocked a second time with 5% NFDM in PBS for 30 min at 37 °C followed by washing five times before the addition of an anti-mouse IgG HRP-conjugate. The secondary antibody was diluted 1:5,000 in PBS and incubated for 1 h at 37 °C. Plates were then washed 10 times in WB and developed with 3,3,5,5-tetramethylbenzidine substrate (Sigma) in perborate buffer (Sigma). The reaction was stopped with 2 M sulphuric acid. Plates were read spectrophotometrically at 450 nm. The anti-Aβ1–42 antibody titre was defined as the reciprocal of the dilution of antisera that produced 50% of the maximum signal detected for that sample.

Histopathology

Mice were overdosed with pentobarbital, perfused with saline and their brains removed. One hemisphere was immersion-fixed in fresh, buffered paraformaldehyde for 24 h. Frozen sections were stained for Aβ peptides by immunohistochemistry[13,19] or for Congo red. The area of frontal cortex occupied by stain was measured with a Videometric V150 image analysis system (Oncor) on a Nikon Microphot FX microscope. Stained regions were measured using HSI segmentation by an experimenter unaware of the subject condition. Both stain intensity and area were measured, although only areas are reported here as this is the convention for Aβ deposits ('amyloid burden'). The results were not qualitatively different when evaluating area, stain intensity or their product (total immunoreactivity[19]). Data were collected from equally spaced horizontal sections for both frontal cortex (anterior to the corpus callosum; 12 per mouse) and hippocampus (10 per mouse).

Received 21 August; accepted 7 November 2000.

1. Schenk, D. *et al.* Immunization with amyloid-beta attenuates Alzheimer-disease-like pathology in the PDAPP mouse. *Nature* **400**, 173–177 (1999).
2. Gordon, M. N. *et al.* Correlation between cognitive deficits and Aβ deposits in transgenic APP+PS1 mice. *Neurobiol. Aging* (in the press).
3. Arendash, G. W. *et al.* Progressive behavioral impairments in transgenic mice carrying both mutant APP and PS1 transgenes. *Brain Res.* (in the press).
4. McGeer, E. G. & McGeer, P. L. The importance of inflammatory mechanisms in Alzheimer disease. *Exp. Gerontol.* **33**, 371–378 (1998).
5. Rogers, J. *et al.* Inflammation and Alzheimer's disease pathogenesis. *Neurobiol. Aging* **17**, 681–686 (1996).
6. Frautschy, S. A. Microglial response to amyloid plaques in APPsw transgenic mice. *Am. J. Pathol.* **152**, 307–317 (1998).
7. Masliah, E. *et al.* Comparison of neurodegenerative pathology in transgenic mice overexpressing V717F beta-amyloid precursor protein and Alzheimer's disease. *J. Neurosci.* **16**, 5795–5811 (1996).
8. Calhoun, M. E. *et al.* Neuron loss in APP transgenic mice. *Nature* **395**, 755–756 (1998).
9. Weiner, H. L. *et al.* Nasal administration of amyloid-beta peptide decreases cerebral amyloid burden in a mouse model of Alzheimer's disease. *Ann. Neurol.* **48**, 567–579 (2000).
10. Bard, F. *et al.* Peripherally administered antibodies against amyloid beta-peptide enter the central nervous system and reduce pathology in a mouse model of Alzheimer disease. *Nature Med.* **6**, 916–919 (2000).
11. Frenkel, D., Solomon, B. & Benhar, I. Modulation of Alzheimer's beta-amyloid neurotoxicity by site-directed single-chain antibody. *J. Neuroimmunol.* **106**, 23–31 (2000).
12. Holcomb, L. *et al.* Accelerated Alzheimer-type phenotype in transgenic mice carrying both mutant amyloid precursor protein and presenilin 1 transgenes. *Nature Med.* **4**, 97–100 (1998).
13. Holcomb, L. A. *et al.* Behavioral changes in transgenic mice expressing both amyloid precursor protein and presenilin-1 mutations: Lack of association with amyloid deposits. *Behav. Gen.* **29**, 177–185 (1999).
14. McGowan, E. *et al.* Amyloid phenotype characterization of transgenic mice overexpressing both mutant amyloid precursor protein and mutant presenilin 1 transgenes. *Neurobiol. Dis.* **6**, 231–244 (1999).
15. Mucke, L. *et al.* High level neuronal expression of Aβ1–42 in wild-type human amyloid precursor protein transgenic mice: Synaptotoxicity without plaque formation. *J. Neurosci.* **20**, 4050–4058 (2000).
16. Hsiao, K. *et al.* Correlative memory deficits, Abeta elevation, and amyloid plaques in transgenic mice. *Science* **274**, 99–102 (1996).
17. Duff, K. *et al.* Increased amyloid-beta42(43) in brains of mice expressing mutant presenilin 1. *Nature* **383**, 710–713 (1996).
18. Diamond, D. M., Park, C. R., Heman, K. L. & Rose, G. M. Exposing rats to a predator impairs spatial working memory in the radial arm water maze. *Hippocampus* **9**, 542–551 (1999).
19. Gordon, M. N., Schreier, W. A., Ou, X., Holcomb, L. A. & Morgan, D. G. Exaggerated astrocyte reactivity after nigrostriatal deafferentation in the aged rat. *J. Comp. Neurol.* **388**, 106–119 (1997).

Acknowledgements

We thank K. Hsiao-Ashe for sharing the Tg2576 mouse line. We thank R. Engelman and U. Owens for superb veterinary services in the maintenance of the transgenic colony. We thank I. Johnson for accounting services associated with the project. This work was supported by grants from the National Institute on Aging to M.G. and D.M., from the National Institute of Allergy and Infectious Disease and the National Heart Lung and Blood Institute to K.U., by the Benjamin Research Trust to D.M. and by an award from the University of South Florida Research Foundation.

Correspondence and requests for materials should be addressed to D. M. (e-mail: dmorgan@hsc.usf.edu).

Induction of vanilloid receptor channel activity by protein kinase C

Louis S. Premkumar & Gerard P. Ahern

Department of Pharmacology, Southern Illinois University School of Medicine, Springfield, Illinois 62702, USA

Capsaicin or vanilloid receptors (VRs) participate in the sensation of thermal and inflammatory pain[1–3]. The cloned (VR1) and native VRs are non-selective cation channels directly activated by harmful heat, extracellular protons and vanilloid compounds[4–8]. However, considerable attention has been focused on identifying other signalling pathways in VR activation; it is known that VR1 is also expressed in non-sensory tissue[1,9] and may mediate inflammatory rather than acute thermal pain[3]. Here we show that activation of protein kinase C (PKC) induces VR1 channel activity at room temperature in the absence of any other agonist. We also observed this effect in native VRs from sensory neurons, and phorbol esters induced a vanilloid-sensitive Ca^{2+} rise in these cells. Moreover, the pro-inflammatory peptide, bradykinin, and the putative endogenous ligand, anandamide, respectively induced and enhanced VR activity, in a PKC-dependent manner. These results suggest that PKC may link a range of stimuli to the activation of VRs.

PKC is a prominent participant in pain signalling. Targeted deletion of PKC-ϵ in mice[10] markedly attenuates thermal- and acid-induced hyperalgesia. In turn, activation of PKC-ϵ potentiates heat-evoked currents in sensory neurons[11,12]. Further, the algesic peptide, bradykinin, potentiates heat responses[11,12], induces depolarization[13–16], and evokes secretion[17–19] from vanilloid-sensitive neurons in a PKC-dependent manner. However, the molecular targets for these effects have not yet been clearly identified. We therefore investigated whether these actions of PKC are mediated by VRs. Rat VR1 was expressed in *Xenopus laevis* oocytes and studied using a two-electrode voltage clamp technique. Treatment with 12-*O*-tetradecanoylphorbol-13-acetate (TPA) to activate endogenous PKC increased the amplitude of currents evoked by capsaicin (Fig. 1a, c), anandamide (Fig. 1b, c) and protons (extracellular pH 5; data not shown). In addition, TPA by itself produced a slowly developing current (Fig. 1a, b) that was not observed in uninjected oocytes (n = 5) or oocytes expressing the NMDA (*N*-methyl D-aspartate) receptor (n = 8). These actions were probably mediated by PKC because no responses were elicited by the inactive TPA analogue, 4α-phorbol (n = 4), and responses to TPA were inhibited by the selective PKC inhibitor[20], bisindolylmaleimide (BIM, 200 nM, Fig. 1c).

Next, we examined whether the current induced by TPA alone was mediated by VR1. In these experiments VR1-expressing oocytes were treated separately with either TPA or capsaicin, to avoid cross-sensitization. Figure 1d shows the response of a TPA-treated oocyte to a series of depolarizing pulses from −80 mV to +80 mV. Outwardly rectified currents were evoked that were similar to those

18/09/03

Elan seeks to resume drug trials

By Ian Guider

ELAN will make another attempt at developing a treatment for Alzheimer's disease a year after a previous drug had to be cancelled.

Elan, which is still emerging from an accounting scandal last year, has filed an application with the US Food and Drug Administration (FDA) to begin trials of a new treatment for the disease.

Last January, Elan had to cancel patient trials of a previous treatment, AN-192, which was found to be causing inflammation of the central nervous system. According to brokers yesterday, the new treatment is a reformulation of AN-192.

Elan said if the FDA had no comments to make on the application it would begin Phase 1 trials later this year with its development partner Wyeth for the drug which is targeted at those who have moderate Alzheimer's.

Analysts believe an Alzheimer's treatment has the potential to be a "blockbuster" drug, with sales of more than $1 billion a year.

The positive news on drug development will be a welcome boost after the company said in July that its potential treatment for Crohn's disease - Antegren - did not have the expected outcome. The company said it was committed to bringing Antegren to the market. Elan reported a return to profitability yesterday, making net profits of $17.3 million for the second quarter of the year.

The company said second quarter revenues were down 46% on last year at $245.5 million but were up 7% when discontinued products were stripped out. The net profit, which equates to 5 cents per share, compares with a loss of $719m a year ago.

Chief Executive Kelly Martin said he is focused on improving the company's financial situation and developing new treatments.

"Elan's second quarter results are characterised by solid progress with our operating plan, including asset divestitures, cost reduction and debt reduction."

Mr Martin added that Elan has almost $1 billion in cash left and further money could come in from the sale of several business units. The company has debt of over $2.3 billion, $1 billion of which must be paid this year.

Elan said that it had made significant headway on reducing its cost base and that its workforce was now down to 2,500 from 4,700 a year ago. Most of the workforce reduction was a result of the sale of several businesses in the past year.

Davy Stockbrokers analyst Jack Gorman said: "Detailed analysis of the underlying operations suggests that Elan remains on track in its recovery plan, and that the ongoing revenue base is intact and tracking a little better than expected.

"A return to substantial profitability will continue to be driven by prospective pipeline approvals."

JCI Scholarly science. Clinical impact.
HOME | CURRENT ISSUE | ARCHIVE | SUBSCRIPTION | ABOUT THE JOURNAL

J Clin Invest. 2003 August 1; 112 (3): 321–323
DOI: 10.1172/JCI200319420

Amyloid β and Alzheimer disease therapeutics: the devil may be in the details

John R. Cirrito[1] and David M. Holtzman[1,2,3]

[1]Department of Neurology, [2] Department of Molecular Biology and Pharmacology, and [3] Center for the Study of Nervous System Injury, Washington University School of Medicine, St. Louis, Missouri, USA

Address correspondence to: David M. Holtzman, Washington University School of Medicine, Department of Neurology, 660 South Euclid Avenue, Box 8111, St. Louis, Missouri 63110, USA. Phone: (314) 747-0286; Fax: (314) 362-2826; E-mail: holtzman@neuro.wustl.edu.

See companion article on p. 415.

See companion article on p. 440.

Abstract

Alzheimer disease (AD) is characterized by the progressive accumulation of amyloid β protein (Aβ) in areas of the brain serving cognitive functions such as memory and language. The first of two separate reports (see the related articles beginning on pages 415 and 440) reveals that intrinsic T cell reactivity to the self-antigen Aβ exists in many humans and increases with age. This finding has implications for the design of Aβ vaccines. The second report demonstrates that a number of FDA- approved nonsteroidal anti-inflammatory drugs are capable of lowering Aβ levels in mice. The work suggests that further testing of the therapeutic utility of these types of compounds for the potential treatment of AD is warranted.

Top
Abstract
Aβ-reactive T cells increase ...
T cells, Aβ, and ... Certain NSAIDs decrease Aβ ...
References

Alzheimer disease (AD) has received a lot of recent attention, particularly in areas related to novel treatments. Recently, the potential therapeutic usefulness of the immune system has become apparent, leading to the question of whether it can be used to directly or indirectly influence AD-related pathology in beneficial ways. Active immunization with amyloid β (Aβ) peptides takes advantage of the immune system to generate antibodies that can somehow decrease Aβ-related pathology in mouse models of AD (1). Similarly, passive immunization involves direct administration of anti-Aβ

antibodies, bypassing the need for an active immune response (2, 3). Since genetic, pathologic, and animal studies suggest that the buildup of Aβ in the brain leads directly or indirectly to cell dysfunction, cell death, and cognitive impairment, increased generation of anti-Aβ antibodies has the potential to prevent or treat AD by decreasing amyloid burden and its consequences in the brain. Though the first clinical trials for Aβ vaccination were halted due to CNS inflammation in a small subset of subjects, active and passive immunization strategies remain a viable potential therapy worth continued exploration. If positive effects can be seen in future trials, it will be important to minimize unwanted toxicity. In this issue of the *JCI*, Monsonego and colleagues (4) further characterize the innate immune response to Aβ in humans, thus revealing important details about how the elderly body reacts to Aβ, and opening new avenues to modify existing vaccination protocols. Also in this issue, Eriksen and colleagues (5) studied traditional NSAIDs that appear to have a nontraditional, COX-independent effect on decreasing Aβ42 production. While these drugs are often used to treat inflammation, they appear to have a novel effect on amyloid precursor protein (APP) cleavage, which is only now becoming apparent and which may be useful in the future as a therapeutic.

Aβ-reactive T cells increase with age

Monsonego et al. (4) found that some healthy, elderly individuals, as well as individuals with AD, contain elevated baseline levels of Aβ-reactive T cells. While the general trend is toward a diminished immune response with aging, this demonstrates a selective increase in Aβ-reactive T cells in older individuals with and without dementia. The reason for this selective expansion of Aβ-reactive T cells in elderly individuals remains unclear. It is often presumed that cognitively normal middle-aged and elderly individuals are similar in that they lack AD pathology; however, Aβ deposition in plaques appears to begin about 10–20 years prior to the onset of even the earliest symptoms suggestive of dementia due to AD (6). This means that some cognitively normal elderly subjects in this study likely possessed aggregated Aβ deposits in the brain, while it is also likely that most middle-aged individuals (younger than age 50) did not have AD pathology. One interesting possibility is that this change in T cell population is a response to the presence of Aβ aggregates even in the absence of dementia. The conformation of aggregated Aβ in AD is predominantly as β-sheets, whereas the soluble Aβ present in blood and cerebral spinal fluid has little or no β-sheet structure. Perhaps, this conformational change in endogenous Aβ stimulates a T cell response. Future studies will be necessary to determine if the peripheral T cell population correlates to CNS pathology or future AD symptoms (i.e., an antecedent bio-marker).

T cells, Aβ, and CNS inflammation

While speculative, individuals with elevated Aβ-reactive T cells may host a greater immune response to an active immunization with Aβ than someone who lacks this T cell change. The positive effects of Aβ immunization in

Certain NSAIDs decrease Aβ ...
References

mouse models (e.g., decreased plaque burden, behavioral improvement) appear to be mediated by antibodies, not the cellular response (7–10). Thus, augmentation of the production of anti-Aβ antibodies is likely to be beneficial. However, in the first trial of active Aβ immunization in AD patients, about 5% of individuals developed a side effect of CNS inflammation. There is evidence that this complication following active Aβ immunization is due to a T cell response (11). It therefore seems logical that minimizing certain aspects of T cell activation would decrease the likelihood of CNS inflammation. Consequently, it may be useful in future vaccination strategies to either exclude subjects that have already demonstrated a substantial T cell reaction to Aβ or to consider these subjects only for passive immunization. Monsonego and colleagues (4) found that the epitopes for Aβ-reactive T cells in humans are primarily amino acids 16–42. Interestingly, however, in studies of active immunization of humans and of mouse models of AD, the primary epitope to which antibodies are generated are amino acids 1–12 (12, 13). Because the cellular and humoral immune responses appear to have distinct, dominant epitopes, perhaps an antigen and adjuvant combination can be designed that favor a humoral immune response over a T cell response.

Top
Abstract
Aβ-reactive T cells increase ...
T cells, Aβ, and ...
Certain NSAIDs decrease Aβ ...
References

Certain NSAIDs decrease Aβ production

Many pathological studies have shown evidence of an inflammatory response (gliosis, increased cytokines) surrounding Aβ deposits in the AD brain. It is thought that this response may result in increased neuronal injury, which suggests the possibility that decreasing this response may be beneficial. In light of this, it is of interest that retrospective, epidemiological studies show that NSAID use is associated with a decreased risk of developing AD. Herein, Eriksen and colleagues (5) further define a different molecular mechanism that may be relevant to this relationship. It appears that certain NSAIDs, potentially in a novel, direct interaction with the γ-secretase complex, can alter APP cleavage and the subsequent species of Aβ produced. Eriksen et al. screened 18 NSAID compounds, including several enantiomers that do not inhibit COX. Interestingly, though structurally similar, these compounds can have different effects on what species of Aβ is produced; some decrease Aβ42, while others decrease Aβ40. The mechanism may be via a direct effect on the γ-secretase complex, which presumably causes a subtle conformational change and alters APP cleavage (Figure 1). In future studies, it will be important to investigate the molecular details of the NSAID/γ-secretase complex interaction. In addition, the drugs most effective in decreasing Aβ levels in humans will need to be determined.

Eriksen and colleagues (5) have focused on decreasing the more aggregation-prone Aβ42 species in order to potentially treat AD. Another potential treatment avenue, however, is to decrease both Aβ42, as well as other species such as Aβ40, the peptide that builds up extensively in cerebral amyloid angiopathy (CAA). If an NSAID compound, or derivative, could be designed to decrease both pathological species of Aβ, it may benefit both diseases. While an important aim is to find a drug to decrease Aβ42, it will be important not to increase Aβ40 levels as a consequence. This could potentially lead to

increased risk for developing CAA and its consequences such as hemorrhage.

These studies provide exciting new insights and avenues for AD treatment by suggesting improvements in current vaccination strategies or by furthering our understanding of how NSAIDs alter Aβ42 production. While it is not going to be easy, there remains much hope that the amyloid hypothesis of AD will be tested and that truly effective therapies for AD can be developed.

Footnotes

Conflict of interest: The authors have declared that no conflict of interest exists.

Nonstandard abbreviations used: Alzheimer disease (AD); amyloid β protein (Aβ); amyloid precursor protein (APP); cerebral amyloid angiopathy (CAA).

References

1. Schenk, D. *et al.* (1999). Immunization with amyloid-β attenuates Alzheimer-disease-like pathology in the PDAPP mouse. *Nature.* **400**, 173–177 [PubMed][Full Text]
2. DeMattos, R.B. *et al.* (2001). Peripheral anti-A beta antibody alters CNS and plasma A beta clearance and decreases brain A beta burden in a mouse model of Alzheimer's disease. *Proc. Natl. Acad. Sci. U. S. A.* **98**, 8850–8855 [Free Full text in PMC]
3. Bard, F. *et al.* (2000). Peripherally administered antibodies against amyloid β-peptide enter the central nervous system and reduce pathology in a mouse model of Alzheimer disease. *Nat. Med.* **6**, 916–919 [PubMed][Full Text]
4. Monsonego, A. *et al.* (2003). Increased T cell reactivity to amyloid β protein in older humans and patients with Alzheimer disease. *J. Clin. Invest.* **112**, 415–422. doi:10.1172/JCI200318104
5. Eriksen, J.L. *et al.* (2003). NSAIDs and enantiomers of flurbiprofen target γ-secretase and lower Aβ42 in vivo. *J. Clin. Invest.* **112**, 440–449. doi:10.1172/JCI200318162
6. Morris, J.C. & Price, A.L. (2001). Pathologic correlates of nondemented aging, mild cognitive impairment, and early-stage Alzheimer's disease. *J. Mol. Neurosci.* **17**, 101–118 [PubMed][Full Text]
7. Morgan, D. *et al.* (2000). A beta peptide vaccination prevents memory loss in an animal model of Alzheimer's disease. *Nature.* **408**, 982–985 [PubMed][Full Text]
8. Janus, C. *et al.* (2000). A beta peptide immunization reduces behavioural impairment and plaques in a model of Alzheimer's disease. *Nature.* **408**, 979–982 [PubMed][Full Text]
9. Dodart, J.C. *et al.* (2002). Immunization reverses memory deficits without reducing brain Abeta burden in Alzheimer's disease model. *Nat. Neurosci.* **5**, 452–457 [PubMed][Full Text]
10. Kotilinek, L.A. *et al.* (2002). Reversible memory loss in a mouse

transgenic model of Alzheimer's disease. *J. Neurosci.* **22**, 6331–6335 [PubMed][Full Text]

11. Nicoll, J.A. *et al.* (2003). Neuropathology of human Alzheimer disease after immunization with amyloid-β peptide: a case report. *Nat. Med.* **9**, 448–452 [PubMed][Full Text]
12. Town, T. *et al.* (2001). Characterization of murine immunoglobulin G antibodies against human amyloid-β1-42. *Neurosci. Lett.* **307**, 101–104 [PubMed][Full Text]
13. Hock, C. *et al.* (2002). Generation of antibodies specific for β-amyloid by vaccination of patients with Alzheimer disease. *Nat. Med.* **8**, 1270 –1275 [PubMed][Full Text]

Figures and Tables

Figure 1. Model of how certain NSAIDs decrease Aβ42 production.